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OBSERVATIONS ON THE LARVAE OF ONCHOCERCA GIBSONI (CLELAND AND JOHNSTON) IN THE SKIN OF INFECTED CATTLE

by

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INTRODUCTION.

This note records the finding of larvae resembling the microfilariae of *Onchocerca gibsoni* in the superficial layers of the dermis of most parts of the body of cattle bearing nodules of *Onchocerca gibsoni* in the brisket.

It must be pointed out, however, that while in the cattle examined, typical nodules containing living worms and larvae of *Onchocerca gibsoni* were present, the presence of other species of *Onchocerca* which, as recorded by Johnston (1921), may occur in Queensland cattle, was not excluded. These species are *O. gutturosa* Neumann and *O. lienalis* Stiles.

Onchocerca gibsoni, described and named by Cleland and Johnston in 1910, has been the subject of extended investigations from most points of view by these and a number of other observers in Australia. To attempt to even summarize this work would be beyond the scope of the present note, and unnecessary, since reviews of it have been published from time to time by Johnston. Only those observations will be referred to which bear more directly on those here recorded. The most recent paper is that of Cilento (1923).

The work in Australia has established the fact that the larvae are either absent or very scarce in the blood. The same is generally true of the closely related parasite *Onchocerca volvulus*, although occasional larvae have been found in the blood by several observers, often probably derived in reality from other tissue. Sharp (1926) has described an interesting but exceptional case, where these microfilariae were constantly present in the blood. But although the microfilariae of *O. volvulus* are absent from the circulating blood of infected African natives, they are present in the dermis outside the blood vessels in considerable numbers. It was this fact, noted by Montpellier and Lacroix (1920), Macfie and Corson (1922), and others, subsequently to most of the Australian work on *O. gibsoni*, which suggested the present observations.

The presence of the larvae of *O. volvulus* in the skin often gives rise to no lesions or macroscopic signs of their presence, although some observers maintain that certain skin affections are caused by them.

More recently Blacklock (1926) has shown that *O. volvulus* is transmitted by *Simulium damnosum*, which abrades the skin in the act of biting, and takes up other material from it besides blood.

Cleland (1914) found larvae of *O. gibsoni* in small thickened patches of the skin of cattle on two occasions, and published photographs of sections of this skin showing the larvae *in situ*. He also found several larvae in the subcutaneous tissues of cattle. It was not at that time recognized, however, that the larvae are present in the skin of the body generally, and in much larger numbers than in other tissues, in which presumably those that are found are merely making their way to the skin.

DETAILS OF OBSERVATIONS.

In July, 1926, a slaughter-yard in Townsville was visited, and a preliminary examination made. Three beasts, all of which had typical nodules of *Onchocerca gibsoni* in the brisket, were examined. A piece of skin, 2 or 3 inches in diameter, was taken from this region of two of the beasts, and two pieces from the third, and in all four specimens larvae were found by immersing the minced skin in physiological saline at room temperature for twenty-four hours and then centrifuging. The larvae seemed identical in appearance with those obtained from nodules, and agreed with the published descriptions of the larvae of *O. gibsoni*.

In these and the further experiments to be detailed below the animals were examined immediately after killing and before skinning. As soon as the presence of nodules was ascertained by palpation and incision the selected pieces of skin were shaved as rapidly as possible and dissected off.

At a second visit to the yard shortly afterwards one beast only was examined, a bullock with typical nodules in the brisket; they were not looked for in other regions. The procedure adopted was as follows: Immediately after killing, small pieces of skin were first roughly shaved, and then dissected off from the different parts of the body detailed in the table given below. This bullock is hereinafter referred to as bullock 1. From each piece of skin twelve circular pieces were punched out by means of a leather punch, and placed in a test tube containing about an inch of physiological saline solution. This was done at the slaughter-yard with as little delay as possible. The diameter of each circular piece of skin punched out was exactly 4 mm., which gives a total area of 150 sq. mm. for twelve pieces. The test tubes were taken to the laboratory, and left at room temperature for twenty-four hours, after which the pieces of skin in each tube were removed from the salt solution, shaken with fresh solution, and the whole of the fluid centrifuged. After removal of the supernatant fluid with a pipette, the deposit

left was well mixed, and a part of it pipetted on to a slide, mixed with a little serum to make it adhere, spread out as a thick layer, and allowed to dry. It was then fixed in rectified spirit, dried, stained with Mayer's haematein, washed and dried, mounted in cedarwood oil, and the larvae counted. The number of larvae obtained from the samples of skin from different parts of the body are given in the table below. In this case the numbers given in the table are the actual numbers counted multiplied by three, since a third was judged roughly to be about the fraction of the deposit examined.

At a third visit to the slaughter-yard, in August, 1926, samples of skin and other parts were again taken from a single bullock in the same manner as before. The work was begun immediately the beast was killed, and all the specimens had been removed within an hour and a half of death; all the pieces of skin within half an hour. Typical nodules were present, as before, in the brisket, and were not looked for elsewhere. Twelve circular pieces of the same size as before were punched from each piece of skin and mucous membrane, and treated in the same way. This bullock is referred to as bullock 2. In this case nearly all the deposit got by centrifuging was examined, so that the number of larvae actually counted approximated to the total number which had emerged from the little discs of skin.

Region of the Sample of Skin or Mucous Membrane.	Number of Larvae obtained from Bullock 1.	Bullock 2.
About 6 inches from the mid-ventral line of the chest, just posterior to the foreleg	327	105
Base of neck, near mid-ventral line	525	235
Over the vertebral column about the level of the fore limb	231	978
Over the vertebral column, about midway between fore and hind limbs	219	91
A point about half-way between the root of the tail and the great trochanter	48	5
Lateral aspect of the thorax, about midway between the fore and hind limbs	15	20
Abdomen, a little to one side of the mid-ventral line, and rather nearer to the pubic symphysis than to the costal margin	87	684
Mid-ventral line between scrotum and tail	6	103
The scrotum	Not examined	71
Dorsal surface of the tail, about a quarter of its length from the root	0	2
Under surface of the tail, about 8 inches from the root	Not examined	7
Forehead, between the eyes	3	7

Region of the Sample of Skin or Mucous Membrane.	Number of Larvae obtained from Bullock 1.	Bullock 2.
Dorsum of nose, half-way between the end of the snout and the eyes	12	1
Outer surface of the pinna	Not examined	1
Inner hairless surface of the pinna	Not examined	4
Outer aspect of the forearm	87	24
Inner aspect of the forearm	Not examined	49
Outer aspect of the carpus	120	94
Inner aspect of the carpus	Not examined	109
Outer aspect of the leg, in the sense of the section of the hind limb supported by the tibia	48	9
Inner aspect of the leg	Not examined	9
Outer aspect of the tarsus	108	33
Inner aspect of the tarsus	Not examined	26
Inferior margin of the nostril just within the junction of the skin and mucous surface	Not examined	60
Mucous membrane of the inner surface of the cheek	Not examined	0
Mucous membrane of the side of the tongue	Not examined	0

Although the figures in this table are considered worth recording, they probably give somewhat imperfect indications of the distribution of the larvae in either beast. The samples were taken from bullock 1 merely with the idea of finding out whether the distribution of the larvae was general over the skin of the whole body. Bullock 2 was intended to be the first of a series to determine the average distribution more exactly with the idea that it might show a relation to the regions most favoured by some of the biting flies. The series could not be continued, and the method would have required improvement. The ratio of the number of larvae which emerged into the saline to the total number in the twelve discs of skin very likely differed considerably for the different samples, for various reasons.

It will be seen, however, that while no close agreement was found between the distribution of the larvae in the skin in the two bullocks, yet in general, with a few exceptions, the regions of abundance and scarcity correspond.

Some observations indicated that the larvae probably emerged from the cut edges of the discs of skin rather than through the epidermis, or by working their way down through the whole thickness of the dermis. In bullock 2 four roughly rectangular pieces of skin, two from the base of the neck and two from the front of the thorax, were cut off from the same two samples from which the punches were made. The perimeter of each of these four pieces was approx. 150 mm., which is also the total perimeter of twelve discs 4 mm. in diameter; on the other hand, the area of each was about 1,500 sq. mm., which is far greater than the area of the twelve discs, 150 sq. mm. Each of these four pieces of skin was immersed

whole in saline in a petri dish, and the number of larvae obtained after twenty-four hours was of the same order as the number from the twelve discs, namely, 355 and 169 from the two pieces from the neck, and 203 and 106 from the two pieces from the thorax.

Trial was not made whether any larvae at all would emerge through the epidermis or through the thickness of the dermis into saline or water in contact only with intact portions of these surfaces, nor were the larvae directly observed in the act of emerging, which could readily be done. Breinl's (1911) results with living beasts, in which larvae were found in water kept in contact with the shaved skin near nodules or in scrapings of the skin, in five experiments out of a hundred, were presumably due to the emergence of larvae lying in the dermis, possibly as a result of the stimulus of shaving or scraping, and perhaps through microscopic breaches in the epidermis caused by these procedures.

It may possibly be that any trauma of the skin, by a biting fly or otherwise, acts as a stimulus to neighbouring larvae to make towards the point, and thus increases their chances of being ingested.

A simple method of ascertaining whether larvae are present in the skin, and gauging their numbers roughly, which has been used for *O. volvulus* in African natives, would probably be of service in living cattle. It consists in pinching up a piece of skin, shaving off a small piece of epidermis with a sharp knife, and then scraping off the tissue fluid mixed with blood which exudes, and transferring it to a slide.

It is possible that an investigation by this method might reveal that, as in the case of *O. volvulus* in man, the percentage of cattle with larvae in the skin is much larger than that with discoverable nodules.

In bullock 2 a piece of the sample of skin removed from the base of the neck was dropped about twenty minutes after removal into picro-sublimate fixing solution, and subsequently serial sections were made, a difficult procedure in skin of this character, but accomplished by Mr. J. W. Fielding, of this Institute. The sections were cut 22 microns in thickness, and stained with Mayer's haematein. Thick sections, provided that a purely nuclear stain is used, give the best view of the larvae; even in sections of this thickness a complete uncut larva was only once seen. It must be emphasized that the brief account which follows of the appearances in these sections may not be true for the living animal in every particular, owing to the possibility of movements of the larvae in the interval between the death of the animal and their killing by the fixative. It would be better to remove the piece of skin the moment the beast has been pithed and plunge it into hot fixative. In the sections the microfilariae were usually more or less coiled, but sometimes extended. They were present only in the superficial layers of the dermis, which is very thick in this part of the body; the deepest noted was about 0·6 mm. from the basal layer of the epidermis. Often they were immediately beneath the epidermis, and were then perhaps more often at the

summits of dermal papillae than in the valleys between papillae. Commonly enough, however, they were some distance beneath the epidermis, and were then often close to the sheaths of hairs or to sebaceous glands. No larvae were seen within the epidermis. The only changes noted in the skin tissues were slight aggregations of cells in the superficial layers of the dermis round the capillaries and small blood vessels. In sections stained with eosin as well as haematein these were seen to include many eosinophile leucocytes.

It is of interest in connection with the mode of transmission to know the number of larvae present beneath a given area of skin, and an attempt was made to determine this in the particular specimen from which serial sections were made. Various methods were tried, of which the simplest was to count the number either of tails or of heads present in a series of sections. It was eventually found that the number lay between 15 and 40 per square millimetre of this skin. As only 235 larvae emerged from the twelve punched pieces of this sample of skin, that is, about 1·6 per square millimetre, it is evident that most of the larvae do not emerge under the conditions of the experiment from discs of skin 4 mm. in diameter. Probably by mincing the skin more finely a much larger proportion of the total would be obtained.

In bullock 2 some of the other tissues and organs besides the skin were examined. About 10 grm. of the tissue, which was not obtained until after the animal had been skinned and partly cut up, was minced with a knife and placed in a large test tube with some physiological saline solution. On arrival at the laboratory the contents of the tube were transferred to a petri dish and more saline added; this was centrifuged after twenty-fours hours, and the larvae in the deposit counted. The findings were as follows:

(1) Superficial fat of the brisket taken quite close to nodules (only about one-third of the centrifuge deposit examined), two larvae found.
(2) Superficial fat further forwards at the base of the neck, not far distant from nodules (only about one-third of the centrifuge deposit examined), two larvae found.
(3) Muscle from the brisket taken quite close to nodules, one doubtful larva.
(4) Muscle of forearm, no larvae found.
(5) Liver (only a fraction of the centrifuge deposit was examined, owing to its bulk), no larvae found.
(6) Lung, containing but little blood, three larvae found.
(7) Heart muscle, ventricle, no larvae found.

DISCUSSION.

The Vector. No discussion of this question, on which so much careful experimental work has been done in Australia, will be attempted here beyond one or two remarks.

Photograph of a larva in a section of skin from the base of the neck of Bullock 2.

The solution of the problem for *O. volvulus* of course increases the probability that a dipterous insect is the vector of *O. gibsoni*. This fly must be one which imbibes not only blood but tissue fluid. For this reason it seems unlikely that a mosquito is concerned, since its slender and delicately wielded piercing organs cause the minimum of laceration and abrasion, and possibly are inserted directly into a small vessel.

The inference from the experiments of Hill, McEachran, and Dickinson (1917) that the vector, if a biting fly, is one which keeps to the open, and does not bite even in an open shed, appears noteworthy.

The Cycle in the Mammalian Host. Nicholl (1914) has shown that larvae can escape through the intact fibrous wall of excised nodules, and doubtless they do so during life. But in the case of *O. volvulus* the larvae are present in the skin of many natives in whom no nodules can be found, and Sharp (1926) has suggested that the formation of nodules is a late phenomenon, and that mature and fertile worms often live free in the tissues. The same suggestion had previously been made by Cleland concerning *O. gibsoni*, but attempts to find the uncapsuled worms have met with little success.

That the route of the microfilariae from the parent worm to the skin is not the blood stream is made probable by the numerous unsuccessful attempts to find them in the blood, while on the other hand a few have been found in subcutaneous tissues on several occasions by Cleland (1914), and in sections of tissues outside blood vessels by Breinl (1913). The fat near nodules in which, as recorded above, a few larvae were found contained very little blood.

SUMMARY.

The distribution of larvae throughout the skin of cattle infested with *Onchocerca gibsoni* is described, and the similarity to the condition found in *Onchocerca volvulus*, in which the method of transmission is known, is pointed out.

In the cattle observed, however, the presence of other species of *Onchocerca* was not excluded.

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MITOCHONDRIAL BEHAVIOUR DURING THE LIFE CYCLE OF NYCTOTHERUS CORDIFORMIS

by

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Recently the presence of mitochondria in protozoa has attracted a great deal of attention among cytologists, and it is the object of this paper to give an account of a further series of observations made upon the mitochondria of a common infusorian, Nyctotherus, and to describe the changes which they undergo during the life cycle of that organism, and the part mitochondria appear to play in cell metabolism.

Methods of Investigation.

In order to observe the mitochondrial behaviour during the life cycle of this infusorian, the rectal contents of starved frogs *(Hyla aurea)*, which are generally infested with *N. cordiformis*, were fixed in Flemming solution without acetic acid, or else in Champy fixative. The organisms were concentrated by centrifuging, and eventually embedded *en masse* in paraffin, and then finally sectioned. These sections, which were cut 4–6μ in thickness, were stained with Heidenhain's iron haematoxylin, and occasionally counterstained in eosin.

On examining these sections with high magnification, the cytoplasm of the protozoan is seen to contain a very large number of rod-shaped bodies, which frequently lie in little groups, having a bacteria-like appearance; but their subsequent behaviour and reactions to certain stains and fixatives show quite clearly that they cannot be symbiotic organisms. When a number of individuals were fixed in absolute alcohol, instead of in osmo-chromic or Champy solutions, these bodies were incapable of being stained by iron-haematoxylin. In this respect they resemble the mitochondria present in other protozoan and metazoan cells, in which these bodies are rendered non-stainable by fixatives containing alcohol. On the other hand, during my previous observations on mitochondria, I found that if various strains of bacteria were fixed for several hours in absolute alcohol, their capacity for being stained with iron-haematoxylin was not visibly affected by this mode of fixation; while mitochondria placed for the same period

in absolute alcohol were quite incapable of absorbing the haematoxylin stain (see figure 5). The non-bacterial nature of the bodies dealt with in this paper is also demonstrated by the following facts: If Nyctotherus be placed for a short time in Ringer's solution, and afterwards sectioned and stained, examination shows that the cytoplasm of the organism contains an increased number of mitochondria (see figure 6), which have evidently been formed by fission of the mitochondria of the normal organism. During my previous observations on Opalina (4), I observed that a similar phenomenon occurred, the Ringer's solution apparently acting upon the mitochondria, stimulating them to increase in numbers, by means of binary fission, the mitochondria subsequently undergoing fusion in places to form large, darkly staining globules. Similar post-mortem effects may be observed with the mitochondria of metazoa (4), merely by allowing the tissue to stand for a short period before fixation. We are justified in concluding, therefore, that these bacteria-like, rod-shaped cytoplasmic inclusions of Nyctotherus are of the same nature as the mitochondria present in other protozoan and metazoan cells.

Behaviour of the Mitochondria.

When the adult Nyctotherus, cut in longitudinal sections, and stained with the Heidenhain process, is examined under high magnifications, the cytoplasm is seen to contain numerous rod-like bodies. If the outer region of the organism is observed, it is seen that these bodies are arranged one behind the other in longitudinal rows, the individual mitochondria attaining a transverse polarity to the axis of the cell (see figure 2); if, however, the deeper parts of the cytoplasm are examined, these mitochondrial bodies are seen to be irregularly scattered throughout the protoplasm (see figure 1).

DESCRIPTION OF PLATE.

All figures (except figure 5) are drawn from material fixed in Osmo-chromic or Champy solutions, and stained with Heidenhain's iron-haematoxylin.

1. Longitudinal section of an adult Nyctotherus, showing the arrangement of mitochondria. The meganucleus and micronucleus and gullet are clearly visible.
2. Oblique section of an adult Nyctotherus, showing the arrangement of the superficial mitochondria, in longitudinal rows. Note the irregular arrangement of mitochondria in the deeper parts of the organism.
3. Longitudinal section of the same, showing three food vacuoles containing mitochondria (not bacteria).
4. Section of Nyctotherus undergoing longitudinal fission. Note the more or less equal distribution of mitochondria into the two developing daughter cells. It is clearly seen that the division of the cell is not accompanied by fission of the mitochondria.
5. Longitudinal section of Nyctotherus, after fixation in absolute alcohol, showing how the mitochondria are incapable of being stained by iron-haematoxylin, after such mode of fixation. Note deeply staining meganucleus.
6. Section of same, showing increase in number of mitochondria after organism has been placed in Ringer's solution. Many mitochondria can be seen undergoing fission.

A further examination of the mitochondria present in both the adult and daughter cells shows that the mitochondria increase by a transverse binary fission, and moreover that fission occurs at irregular intervals. Fauré-Fremiet (2), in some of his former works dealing with mitochondria in the protozoa, states that division of the cell is accompanied by a simultaneous fission of the mitochondria. But I have previously found that in Opalina (4) and in Paramoecium (5) no such phenomenon occurs. I am now able to report that in Nyctotherus also mitochondrial division does not synchronize with nuclear fission (see figure 4). As in Paramoecium and Opalina, so I find that in Nyctotherus there occurs a gradual increase in their number during the growth of the individual, and the easily observed fact that the number of mitochondria present in the young daughter cells is far less than the number present in the adult cells does not add weight to the statement of Fauré-Fremiet.[1]

The Function of Mitochondria in Cell Metabolism.

Quite recently Marston (8) has shown that the behaviour of the mitochondria towards azine dyes is exactly identical with the reactions which the proteolytic enzymes undergo in the presence of these substances, and he is led to the conclusion that they contain concentrated within them the synthesizing enzymes of the cell, their action being to build up protein at their surface.

In earlier years Regaud and Mawas (9) suggested that in the salivary glands the secretory granules arose from the mitochondria, and pointed out their relationship to enzymes; while Arnold (1) in 1912 showed that the zymogen grains in the pancreas arose from cell mitochondria. I have also observed (6) that the zymogen granules present in the pancreas of guinea-pigs arose from the filamentous mitochondria, and were secreted into the acinar ducts, where they eventually became dissolved. These observations strongly suggest the enzymatic nature of the mitochondria.

Later observations have shown that in Paramoecium (5) and in Amoeba (7) the mitochondria come into contact with the food particles within the food vacuoles, and apparently bring about disintegration of the food.

Here we seem to observe a process of intracellular secretion of enzymes in organisms which exhibit only intracellular digestion, similar to the process of extracellular secretion as observed in gland cells. Furthermore, in Opalina (4) I have observed that a large granule may develop in close connection with each mitochondrium, this granule evidently being a food storage substance, the result of local synthetic activity occurring through the concentration of diffusible foodstuffs that have penetrated the cuticle of the organism.

[1] It is interesting to observe that Hegner and Taliaferro (3) state the Nyctotherus increases only by means of a transverse binary fission, while two of my cut and stained sections show that these organisms are also capable of reproduction by longitudinal fission (see figure 4).

Studies on Nyctotherus have shown that in this form synthetic activity of the mitochondria cannot be detected, and it is quite possible that Opalina may be a more favourable object for observing this formation of storage grains.

Nyctotherus, like Paramoecium, feeds in a truly holozoic manner, and a process of intracellular digestion similar to that which I have already described in Paramoecium may be observed also.

Cut and stained sections of Nyctotherus under high magnifications show that rod-like mitochondria, which can easily be distinguished from bacteria, are present in the food vacuoles (see figure 3).

In a recent paper dealing with the function of mitochondria in living amoebae, I observed that when a foreign organism is engulfed by the amoeba, a number of mitochondria adhere to the surface of the food; finally a vacuole is formed around the food particles, and the mitochondria gradually dissolve and the food disintegrates.

In amoebae I have never observed the extrusion of mitochondria into these vacuoles, but the mitochondria always adhere to the surface of the food first, and it is only subsequently that a vacuole is formed around the food particle. In fact, in amoeba there is a good evidence that the mitochondria do not penetrate into the vacuoles, but the vacuole is always formed after the mitochondria have adhered to the captured food.

Living amoebae are particularly favourable objects for the study of this, as the movements undergone by the organism are slow, and permit careful observation.

Nyctotherus, on the contrary, is far too active to permit the making of observations on this question in the living organism. As in Paramoecium, so in Nyctotherus, it must at present be left an open question as to how the mitochondria come to lie within the food vacuoles; of their presence there can, of course, be no doubt, and in making the observations no confusion between mitochondria and engulfed bacteria has arisen. In living amoebae, as I have previously reported (7), mitochondria may lie close to the wall of the vacuoles, where, undergoing marked Brownian movement, they even strongly indent the surface of the vacuole; nevertheless, I have never observed them to break through into it.

In analogy with what we find in amoeba, therefore, it seems more probable that the mitochondria are not thrown into the food vacuoles, but that the vacuoles are only subsequently formed around food particles to which the mitochondria have previously adhered.

SUMMARY.

1. Mitochondria are present in Nyctotherus during all stages of the life cycle; they increase by transverse binary fission at irregular intervals during the life history of the organism, and there is no relation between nuclear division and fission of mitochondria.

2. Mitochondria are found within the food vacuoles of Nyctotherus. This is regarded as a direct demonstration of the origin of digestive enzymes from mitochondria.

I am greatly indebted to both Professor W. E. Agar and to Dr. O. W. Tiegs for their valuable suggestions. This investigation was carried out while holding a Science Research Scholarship in the Department of Zoology of the University of Melbourne.

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ON THE RELATION OF MITOCHONDRIA TO THE NUCLEUS

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INTRODUCTION.

This small piece of work has been carried out in order to investigate the relation of the mitochondria to the nucleus. Various authors have frequently drawn attention to the peculiar aggregation of mitochondria round the outer surface of the nuclear membrane in many cells, while Rikita Honda, in a recent paper (3), states that mitochondria are actually traceable through the nuclear membrane. As this conclusion of Rikita Honda is not in accord with my own observations, I have considered the subject deserving of a further investigation.

MATERIAL AND METHODS.

In order to investigate the phenomena of the mitochondria in the nuclear region, both protozoan and metazoan tissue was used. When dealing with the protozoa, two common types of Infusoria, namely, Nyctotherus and Paramoecium, fixed in either a Flemming solution without acetic acid, or else in a Champy fixative, were concentrated by centrifuging, brought up through the alcohols, cleared, eventually embedded *en masse* in paraffin, and finally sectioned. The sections were cut 3–5μ in thickness, stained with Heidenhain's iron-haematoxylin, and occasionally counterstained with eosin.

The metazoan material used in this research was the submaxillary gland obtained from the adult albino rat. It was treated by exactly the same methods as regards fixation and staining as the protozoan material, the only difference being that the sections were cut somewhat thicker, having a thickness of 6μ.

If prepared sections of either Nyctotherus or Paramoecium are examined under high magnifications, the cytoplasm of the organism is seen to contain a large number of rod-like bodies, and in cases when a section has been cut through the middle of the meganucleus of the organism, these bodies are generally seen to be aggregated round the outer surface of the nuclear membrane, in a manner

which will be described more fully in a later portion of the paper. These rod-shaped bodies certainly present a bacteria-like appearance, but their subsequent behaviour to certain stains and fixatives shows quite clearly that they cannot be foreign organisms. A ready test for mitochondria is available, whereby one may distinguish them from ordinary bacteria. If bacteria are exposed for several hours in absolute alcohol they afterwards stain up sharply with iron-haematoxylin, while mitochondria are rendered non-stainable even after quite a short exposure. In the case of Nyctotherus and Paramoecium this test was applied, and the mitochondria present in both these infusorians were rendered non-stainable by the Heidenhain process (see figure 5). In fact, mitochondria are incapable of absorbing the stain if fixed in a solution containing even a small proportion of either absolute alcohol or acetic acid, while bacteria are not visibly affected by this treatment.

If cut and stained sections from the submaxillary gland of the albino rat are examined, numerous long filamentous bodies, together with a large number of small spherical bodies, may be seen in the cytoplasm of the cells with great clearness, and the filamentous bodies may occasionally be seen extending over the nuclear membrane. These bodies, when treated with either absolute alcohol or acetic acid, are also rendered non-stainable; and, in fact, respond in exactly the same way as those other rod-shaped bodies present in both Nyctotherus and Paramoecium. From these considerations I believe these cytoplasmic inclusions to be of the same nature as the mitochondria present in other animal and plant cells.

OBSERVATIONS AND DISCUSSION.

When Nyctotherus and Paramoecium are examined in longitudinal section after staining with the Heidenhain process, the cytoplasm of these organisms is seen to contain a number of minute rod-shaped mitochondria scattered through the cytoplasm.

If, however, a section cut through the middle of the meganucleus be examined, it will be observed that rod-like mitochondria are arranged round the nucleus,

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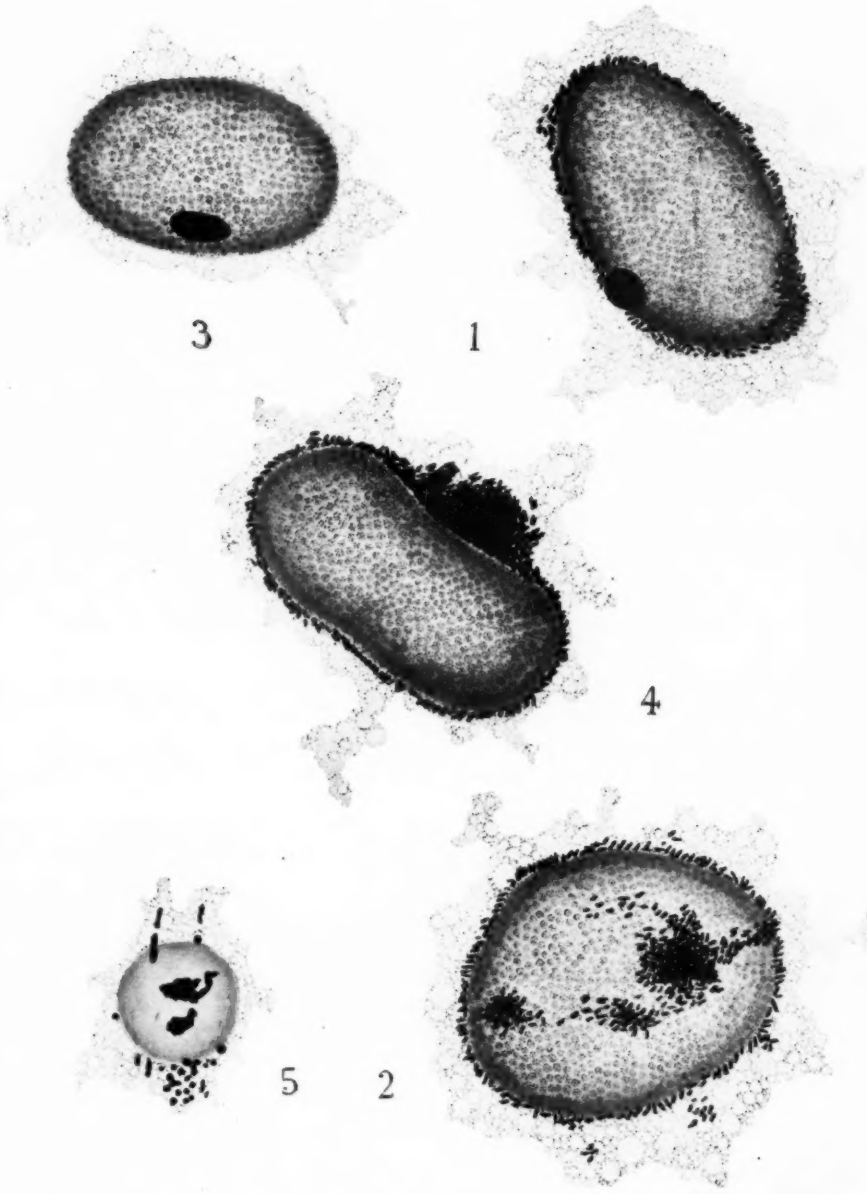
REFERENCE TO PLATE.

All figures (except figure 3) are drawn from material fixed in either Flemming's solution without acetic, or else in a Champy fixative, and stained with Heidenhain's iron-haematoxylin, and occasionally counterstained in eosin.

1. Oblique longitudinal section, cut through the middle of the meganucleus of Nyctotherus. Note mitochondria arranged around the nucleus, lying quite close to and in intimate contact with outer surface of nuclear membrane. Micronucleus clearly visible.
2. Surface section of same, showing mitochondria in close contact with surface of nucleus.
3. Absolute alcohol fixation of same demonstrating absence of mitochondria after such treatment. Micronucleus clearly visible.
4. Longitudinal section of meganucleus of Nyctotherus, showing a large aggregation of mitochondria in front of and in close contact with outer surface of nucleus.
5. Longitudinal section of nucleus from a cell of submaxillary gland, showing filamentous mitochondria lying across outer surface of nuclear membrane.


3
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2

lying quite close to and apparently in intimate contact with the outer surface of the nuclear membrane (see figure 1).

I further observed that if a surface section of the meganucleus be examined numerous mitochondria were to be seen in close contact with the surface of the nucleus, and often covering the whole surface. This interesting phenomenon may at first give the impression that the mitochondria are actually traceable through the nuclear membrane, but a further and more detailed observation shows quite clearly that these bodies only lie in intimate relation with the outer surface of the membrane, and do not occur within the nucleus (see figure 2).

Rikita Honda, however, in a recent paper dealing with mitochondrial behaviour in the submaxillary gland of the albino rat, states that the mitochondria are actually traceable through the nuclear membrane of the epithelial cells. On reinvestigating this work, I find that stained and cut sections of the submaxillary gland of these rats, prepared by the usual mitochondrial technique, show filamentous mitochondria occasionally lying across the nucleus in close contact with the outer surface of the membrane, in much the same way as do the mitochondria present in both Nyctotherus and Paramoecium. In no case, however, have I observed a similar phenomenon to that described by Rikita Honda, where the mitochondria actually occur within the nucleus.

When examining the acinar cells of the pancreas of the guinea-pig, I have also noticed mitochondria lying in a similar fashion in intimate relation with the outer surface of the nucleus.

Recently many authors have drawn particular attention to the various aggregations of mitochondria which occur in contact with the outer surface of the nuclear membrane in many types of cells. While dealing in particular with Nyctotherus, my attention was drawn to a dense mass of granules frequently occurring in front of and in close relation with the meganucleus (see figure 4). Later I observed that Hegner and Taliaferro (2), as well as Minchin (5) have also remarked upon this fact. The dense granular accumulation may be seen quite clearly as a highly refractive mass in the cytoplasm of the living cell. By applying the usual mitochondrial technique to these bodies, I discovered this granular mass to be an aggregation of mitochondria, presenting a similar appearance to that shown in the figures of Harvey (1) and other authors in various cells, where aggregations of mitochondria collect round the outer surface of the nucleus. Harvey, who made a study of mitochondria in the oogonium of *Lumbricus*, describes and figures a similar dense mitochondrial mass in close contact with the outer surface of the nuclear membrane.

So far as I am aware at present, no author has interpreted the occurrence of this frequent phenomenon. While dealing with this problem it is of interest to note that Mayer, Rathery, and Schaeffer (4) have recently contended that mitochondria are composed largely of phosphatides which contain fatty acid radicles; and moreover, it is known that phosphatides, like other fatty substances,

accumulate at the phase boundary of the cytoplasm, owing to their capacity to reduce surface tension, according to the Gibbs-Thomson law. It is possible that the accumulation of mitochondria at the nuclear-cytoplasmic interface may be related to this property of their phosphatidal components. Such a conclusion would also help to explain the frequent occurrence of mitochondria round the vacuolar membranes, and also round the peripheral region of many cells.

SUMMARY.

1. Observations show that mitochondria are not traceable through the nuclear membrane in the epithelial cells of the submaxillary gland, as has previously been stated by Rikita Honda.

2. Dense aggregations of mitochondria about the outer surface of the nucleus, such as is seen in many cells, may possibly be a surface tension phenomenon, and is apparently dependent upon their phosphatidal nature.

This investigation was carried out while holding a Science Research Scholarship in the Department of Zoology of the University of Melbourne.

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THE STRUCTURE OF THE NEURONE JUNCTIONS IN SYMPATHETIC GANGLIA, AND IN THE GANGLIA OF AUERBACH'S PLEXUS

by

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The present investigation is part of an attempt to extend to the remainder of the nervous system the conclusions I have previously reached for the cells of the spinal cord, namely, that the neurone junctions occur, not as discontinuous synapses which are closely applied to, but are not continuous with the body of the nerve cell, or ramify among its dendrites, but that, on the contrary, they penetrate the nerve cell, and become identical with its neurofibrils.

General Remarks.

The minute structure of neurone junctions within the nervous system has been the subject of numerous investigations within the past fifty years. Before the introduction of the special methods of Ehrlich, Golgi, and Cajal, the opinion of Gerlach prevailed, namely, that the central nervous system was composed of a meshwork of nerve cells, the nerve cells anastomosing with one another by way of their dendrites.

With the introduction by Golgi, about 1873, of the silver-chromate impregnation method, the modern neurone doctrine arose. Golgi observed the presence of long fibres within the central nervous system, but declared that there occurred a true neurone continuity, the axon collaterals of one neurone being continuous with the collaterals of other nerve cells.

This theory of collateral continuity was opposed by Cajal, by Forel, by Kölliker, and by Retzius, who concluded that the network of Golgi was in reality merely a feltwork, and that no direct anastomosis of collaterals therefore occurred. On the contrary, it was found, as Arnold had already observed for the frog's sympathetic, in 1865, that the nerve fibres underwent terminal ramifications, which were believed to be applied to the body of other nerve cells. Similar observations by Ehrlich (1886) and others led in 1891 to the formulation by

Waldeyer of the neurone theory, in which the neurones were conceived as the morphological units of which the nervous system was composed. These neurones were not continuous with one another; the endings were by contact—there was not continuity, but contiguity, and to this break in the reflex chain the features by which conduction of a reflex arc is distinguished from conduction along the nerve fibre were attributed. Based on Cajal's conception of the ramifying terminations of nerve fibres embracing the body and dendrites of nerve cells, but not penetrating them, Michael Foster and Sherrington in 1897 proposed the term "synapse" (synapsis), from *σύν*, and *ἅπτω* (clasp).

Although the theory of neurone discontinuity has received the strong support of numerous investigators, Auerbach (3), Semi Meyer (20), Dogiel (10), Retzius (21), Lenhossek, M. Heidenhain (13), and more recently Bartelmez (5), yet there are a number of investigators who have strongly opposed it. In 1897 Apathy (1) described a neurofibril continuity in the nerve cells of invertebrates. Neurofibrils from the sensory neurones communicated with neurofibrils from the motor neurones either directly or by way of an anastomosing neurofibril network (diffuses Elementargitter). This observation was confirmed and extended by Bethe (7). In the leech and earthworm, according to Bethe, neurofibrils emerge from the trunks of the sensory neurones, and either penetrate directly into the motor neurones, or first unite with a small number of other, apparently sensory, neurofibrils to form a "fibrillengitter," from which a single neurofibril emerges, and penetrates the motor cells. While in these worms the indirect passage of neurofibrils via the "fibrillengitter" is believed to occur in the minority only of cases, in the crab *Carcinus* Bethe believes it to be the predominating mode of neurone junction.

Retzius, on the contrary, states that he has examined Apathy's own preparations, and can find no trace of a "fibrillengitter"; there occurs a twist-work, but no anastomosis. The nervous system of vertebrates Bethe believes to be somewhat differently constituted. On the surface of the nerve cells and dendrites occurs a network, first observed by Golgi, and termed the Golgi net by Bethe. Bethe regards this superficial Golgi net as being nervous in nature and homologous with the "fibrillengitter" of the invertebrate nerve cord. Into it pass the fibrils from the sensory neurones, and from it pass fibrils which enter the nerve cells and become its neurofibrils. There is thus a neurofibril continuity via a superficial nervous Golgi net.

Cajal regards the Golgi net as a mere coagulation artefact. In a recent paper Marui (19) believes it to be nervous in nature, but an examination of his figures reveals a most obvious coagulated "foam." I have recently observed the Golgi net on the surface of nerve cells from the spinal cord; I find that it shows a special tendency to occur in regions where the intercellular matrix has undergone "frothing," and no evidence whatever is available to show that it is anything but a very obvious artefact.

Held (14) has described a "pericellular nerve terminal net" on the surface of the nerve cells, quite distinct from the Golgi net. It has apparently arisen from the fusion of neurones. The "axospongium" of the nerve terminals is described as "loosening," and having "neurosomes" densely embedded in it. These end-buds he describes as having a definite connection with the cell body. With the use of the reduced silver method, Held in 1905 maintained the continuity of the fibrils of the axons and the neurofibrils (15). Holmgren confirms this (16). Bielschowsky (8) describes a direct continuity between the end-knobs and the protoplasm of the Purkinje cells of the cerebellum, and attributes Cajal's discontinuity to an incomplete impregnation of the end of the fibre.

More recently Bartelmez (5) describes a contact relationship of fibres from the vestibular nerve with the giant Mauthner cell of fishes and tailed amphibians, while Marui (19), using the same material, finds that the neurofibrils from the fibres of the vestibular nerve actually penetrate the body of Mauthner's cell, thus essentially confirming Held's conclusion on this point. In a recent paper F. M. Ballantyne (4) finds that the fine fibres which traverse the grey matter of the spinal cord in *Lepidosiren* penetrate the nerve cells, without the formation of end-buds, and are continued into the cell as its neurofibrils.

In my own studies on the nerve cells of young mammals (rabbit, guinea-pig, cat) I find (23, 24) that the fine fibres (collaterals) that are to be observed in thousands in the grey matter of well-impregnated spinal cord sections converge upon the dendrites of the nerve cells, penetrate them, and are continued within these as the neurofibrils of the cell. In the centre of the cell these fibrils appear to anastomose, and this is apparently the seat of the integration. Fusion of neurofibrils in the dendrites, as described by Kato (17) and others, is probably an artefact, due to the fibres sticking together during the killing of the protoplasm. In these studies no indication of ramifying synapses embracing the nerve cells, or spread out among its dendrites, was observed. It was pointed out that most of the evidence obtained by Cajal for the existence of such structures was invalid; and while it was not possible definitely to exclude the existence of such synapses, yet the predominant type of neurone junction was one of direct continuity; not, indeed, through a superficial Golgi net as described by Bethe, but by the direct entrance of the fibrils into the dendrites of the cells. Occasionally a fibril penetrates the body of the nerve cell directly, but this appears to be quite an exceptional condition, and, so far as I could see, only the dendrites are usually involved.

The Neurone Junctions in Sympathetic Ganglia and in the Ganglia of Auerbach's Plexus.

When we attempt to extend these observations to the sympathetic system special difficulties are encountered. The reduced silver method, which gives such

excellent and consistent results with the spinal cord, is far less satisfactory when applied to the sympathetic ganglia. The fixation is often bad, and neurofibril impregnation often does not take place. Under these circumstances one finds in practice that it is best to make a large number of preparations, and discard all those which are inferior. What underlies this irregularity in the staining is not known; possibly it depends upon the density of the fatty tissue that often surrounds the ganglia. The second difficulty that is encountered is that the collaterals from the neurones that enter the ganglion do not run approximately in one plane, as they usually do in the cord, but on the contrary run a most irregular course through the ganglion. While, therefore, in the cord we are able to trace bundles of collaterals, from the white matter almost to their termination in the grey, bundles of collaterals are so ill-defined in the sympathetic ganglia that this is not possible.

The observations recorded here have been made with the use of Cajal's reduced silver method, and the methylene blue technique. For staining the thoracic ganglia I have used Ehrlich's method of injecting a weak solution of methylene blue through the dorsal aorta of a freshly killed animal; for the ganglia of Auerbach's plexus Dogiel's immersion method gives excellent results. With the silver method it is advisable always to use young animals, as these seem to give a much better neurofibril impregnation. For material I have used thoracic ganglia from newly-born rabbits, guinea-pigs, and kittens, unborn calf, and ganglia from fully grown frogs, lumbar and coeliac ganglia from adult men, Auerbach's plexus in young rabbits and guinea-pigs, and in fully grown frogs.

Since the structure of the peripheral intestinal plexuses, as Dogiel has shown (11, 12), is closely similar to that of the sympathetic ganglia, I have thought it advisable to supplement the study of the latter by a careful examination of Auerbach's plexus. This is facilitated by the fact that these widely dispersed plexuses obviously offer ideal conditions for studying the structure of neurone relationship. A description of the human sympathetic ganglia will be deferred till the end of the paper, since these ganglia, at first sight, differ in some important respects from the sympathetic ganglia of animals.

Spinal medullated nerves enter the sympathetic ganglia via the white rami: within the ganglia they appear either to terminate in a large arborization, or they may pass right through the ganglia, usually giving off one or more collaterals to it, and finally terminate in some other ganglion higher or lower in the chain. These arborizations have been observed by Cajal (9), Kölliker, van Gehuchten, and others. In drawings which Cajal has given, they are shown ramifying over a large part of the ganglion. Thus, in figure 550 of Cajal's book, axis cylinder arborizations are shown traversing a region of the ganglion four or five times that which would be occupied by a single nerve cell and all its dendrites. In figure 554, taken from a young chick, the collateral arborizations occupy about

the same area as that occupied by a single nerve cell and its dendrites. What is the relation of these collaterals to the sympathetic cells? It has been pointed out by Cajal that a terminal arborization comes into contact with a large number of sympathetic cells, but the actual relation of the arborization to the nerve cells he did not observe.

In the cells of the spinal cord, as I have previously found, the predominant type of neurone junction is in the form of collaterals penetrating the cell dendrites. Although Cajal has described ramifying terminations of nerve fibres ending as pericellular expansions upon the surface of posterior horn cells or their dendrites, I have not been able to observe these. In the sympathetic cells, on the contrary, their presence may be definitely affirmed. They were first observed in the frog's sympathetic by Beale in 1863 (6), and by Arnold (2), long before the introduction of special staining methods. In this situation they have also been described by Aronson and by Retzius in the rabbit; Warfwinge (26) has observed them in the frog; they have also been found by Cajal and by Kölliker, the latter observer finding sometimes that more than one fibre may be involved in this pericellular termination. Excellent illustrations of such terminations occurring in sympathetic ganglia of fish, amphibia, reptiles, birds, and mammals, have also been given by Huber (18). Dogiel (11) has likewise observed these pericellular terminations on the cells of Auerbach's plexus. In this case they arise from fine medullated fibres, and are therefore probably derived from the vagus nerve, and not from the sympathetic.

Using the reduced silver method, I have had no difficulty in rendering these pericellular terminations visible in the frog (see figure 12). In this animal the fibre, on approaching the cell, winds itself spirally round its axon, as Beale and Arnold observed over sixty years ago, and usually ramifies upon its surface. Occasionally the ramification does not seem to occur, and the fibre winds itself round and round the cell (figure 12). In figure 15 is shown a nerve cell from Auerbach's plexus, with two pericellular endings upon its surface.

It must be admitted that we have here a true pericellular termination of a kind whose existence in the spinal cord cannot yet be accepted. Lest it be suspected that this in itself contradicts my previous observation on the spinal cord, it may be pointed out that the frog's sympathetic is in this respect peculiar. The ganglion cells in the frog are devoid of dendrites; consequently a neurone junction via cell dendrites cannot occur. I find, therefore, in agreement with Ranson and Billingsley (21) that this is the only type of neurone junction occurring in the sympathetic ganglia of the frog. Although Huber (18) has found similar terminations in mammals, it will be pointed out below that in this class conditions are more complex, and a definite penetration of collaterals into the cell dendrites constitutes the predominant type of neurone junction here, a type of junction, in short, of the same kind as I find in the spinal cord. This conclusion is supported by the observations of Ranson and Billingsley, who find

a highly developed intercellular plexus between the ganglion cells of mammals, a plexus which degenerates upon section of the preganglionic fibres. What the relation of this enormous number of fibrils to the ganglion cells is, they do not directly observe, but believe that there can be little doubt that synaptic relations exist between them and the dendritic ramifications. It will be shown below that this "synaptic relationship" is of the same type as that occurring in the spinal cord, namely, that the collaterals penetrate the dendrites and become the neurofibrils of the cell.

Although, it is true, these preparations from the frog clearly reveal a definite pericellular termination on the surface of the sympathetic cell, they do not enable us to say whether it is a case of contact or of actual fusion of the two neurones. In surface view the terminal expansion certainly seems to lie upon the outside of the cell, and Ehrlich many years ago observed the fine terminal fibre, stained with methylene blue upon the surface of the cell, and not apparently entering its substance. These observations, however, throw but little light upon the question; perfect preparations of the endings of motor nerves on "striated" muscle fibres, when seen in surface view, seem to reveal the end-plate as merely lying upon the surface of the fibre; nevertheless, it has been found, especially by Boeke, that these motor nerve fibres have penetrated below the fibre, and have entered its substance. The case of motor endings on muscle, indeed, is of particular interest when we recall the discussion which has prevailed in the past concerning the hypolemmal or epilemmal nature of these endings. The case of the motor nerve endings upon muscle fibres illustrates the futility of the argument, often advanced, that a neurone discontinuity must exist, on the ground that a pericellular ending may stain sharply with methylene blue or with silver-chromate, while the cell upon which it ends remains unstained; for in the case of the motor endings precisely the same may be observed. If a frog's sartorius muscle be immersed in a little methylene blue, and kept under observation, it is found that many of the nerve fibres and their terminal plates stain a deep blue, while the muscle fibre remains unstained; but one also observes many muscle fibres which stain strongly, but in which the motor end-plate remains unstained; yet we would not be justified in concluding that therefore the motor nerve merely lies in contact with the muscle fibre. Similarly, the teaching of Löwit, that the motor endings on plain muscle fibres are intraprotoplasmic (hypolemmal) in position has been contradicted by Cajal, Retzius, Arnstein, and others, nevertheless, it has been clearly shown by Boeke, and by myself (25), that Löwit's original contention is correct.

We conclude, consequently, that while in the spinal cord pericellular terminations have not yet been satisfactorily demonstrated, in the sympathetic cells they certainly occur; at the same time the observations are insufficiently precise to enable us to determine whether the endings have penetrated the cell or whether they merely lie upon its surface. Analogy with motor endings on muscle certainly suggests that the former is the more probable.

But we cannot necessarily conclude from this that the sympathetic cells are fundamentally different from the cells of the spinal cord. When preparations of mammalian sympathetic cells, made by the reduced silver method, are examined, it is found that the neurofibrils which form a very open network within the cells emerge from these, and traverse long distances of the ganglia as naked extracellular neurofibrils. This is clearly shown in figures 1, 8, 9, 10.

Numerous figures of the neurofibril organization of the sympathetic cells have been given by Cajal, by Warfwinge, and by others. These drawings reveal an extraordinarily dense meshwork of the very finest fibrils, pervading the whole nerve cell and its dendrites. I have obtained preparations showing the same condition, and have observed it also in the posterior root ganglia. These neurofibrils have a very different appearance from the neurofibrils which the same method can reveal with such astonishing clearness in the cells of the spinal cord. I strongly suspect that we are concerned here with an artificial product of the fixation of the cell protoplasm, and that the neurofibrils are in no way the same as the neurofibrils revealed in the spinal cord cells. They appear to be one of the numerous artefacts obtained with the use of an as yet very imperfect technique. I find, on the contrary, here and there among the many cells examined well-fixed cells showing neurofibrils of precisely the same kind as are found in the spinal cord cells. Such a cell is shown in figure 1. Lest it be urged that here, too, we are victims of an artefact, it may be remarked that the main evidence in favour of regarding these neurofibrils as definite cell entities is that they can be traced as independent fibrils along the dendrites, and then emerge and traverse the ganglia as naked extracellular neurofibrils.

Now when these naked fibrils are traced outwards from the cells we find that they either enter the bundles of other similar or thicker fibrils—in which case they cannot any longer be individually followed—or, as often happens, after tracing them for considerable distances, they emerge from the plane of the section. Frequently, and especially is this the case at the surface of the ganglia, the cells are devoid of dendrites, and the neurofibrils emerge directly from the cell body and traverse the ganglia as naked neurofibrils (figure 9).

Now if thick sections of sympathetic ganglia, prepared by the Ehrlich method, or the reduced silver process, be examined, we can observe many cases of axons branching considerably in the substance of the ganglion, probably mainly arborizing collaterals from medullated spinal nerves (see figure 2). The fine fibrils which thus arise can be followed often for great distances. They are usually of extraordinary delicacy, and much finer than the spiral fibre which forms pericellular endings around the ganglion cells. I have examined many hundreds of these fine collaterals, almost always with the same result. Like the naked neurofibrils that emerge from the nerve cells, and to which they are similar in appearance, they can be traced either into a bundle of fibrils, within which they cannot any longer be followed, or they leave the plane of the section. The

path of the fibres within the ganglion is, however, so irregular that we are even less likely to trace such a collateral to its termination than we are in the case of the spinal cord, where, indeed, it is difficult enough. During a prolonged examination of many hundreds of such collaterals, extending over a period of several weeks, I have succeeded in finding only a few cases where such a collateral could be traced to its ending. In these cases, some of which are shown in figures 3, 4, and 5, the collateral was a very short one, and not so irregular in its path as to emerge from the plane of the section before reaching its destination.

A careful study of the minute structure of the sympathetic ganglion seems to show, therefore, that in the ganglion we have two types of neurone junction; there is a direct neurone continuity similar to that found in the cord, in which fine branches from the arborizing collaterals travel an irregular path in the substance of the ganglion, and penetrate the dendrites of the cells; or if, as often happens in sympathetic ganglion cells, dendrites are few in number, directly enter the cell body (this is the method by which the intercellular plexus of Ranson and Billingsley ends); and secondly, there occur pericellular endings enveloping the cells, and probably penetrating its substance, though this has not yet actually been observed. The latter occur in mammals (Huber), and constitute the sole type of neurone junction in the ganglia of the frog.

Lest it be urged that there is little real evidence in favour of the former type of junction, it may be pointed out that though this has been but rarely observed *directly*, we are forced to this conclusion on certain grounds. Firstly, there occurs seldom more than one pericellular ending upon the "surface" of the nerve cell. A convergence of neurones upon a final common path would not therefore seem to occur, and integration would not be possible.[1] Secondly, in well-fixed reduced silver preparations we find that the fine branches of the collateral arborizations are much too thin to be identical with the fibre which forms the pericellular ending. There must occur, therefore, a neurone junction quite apart from the thick pericellular ending. The fact that this second type of neurone junction, which I regard as the predominant one for the sympathetic ganglia of the mammal, is so extraordinarily difficult, if not almost impossible to demonstrate *directly*, cannot be taken as an argument against its existence; for such a junction could only be directly revealed if the fibrils ran a very regular course within the ganglia, and this they certainly never do. This much is definite: that for almost every axon collateral that we observe under the microscope no real ending can be found.

In methylene blue preparations of sympathetic ganglia, in cases where only a few cells have been stained, it is possible to observe fine fibrils "leaving" the nerve cells (figure 7). Of these one is the axon, and is usually recognized by

[1] In the case of the frog it must be admitted, however, there is no evidence that such integration occurs, for, as a rule, only one pericellular ending is seen on the surface of a single nerve cell.

the collaterals that arise from it. What are the other very fine fibrils that seem to grow out from the cell? If we trace them away from the cell they either emerge from the plane of the section or gradually fade out. This does not signify a true ending of the "dendrites," for their ending is never a sharp one, but is due to a gradual failure of the stain to impregnate them further. These "dendrites" are indistinguishable in appearance from fine nerve fibres, and they cannot be shown to have any relation to other nerve cells. Nevertheless, to dismiss them with the name "dendrite" does not seem to throw much light upon their real nature; they are quite indistinguishable from fine nerve fibres in appearance, but they cannot be shown to have any relation with other nerve cells. Furthermore, there does not seem any reason to homologize them with the Type 11 cells found by Dogiel in the myenteric plexus, and believed by that investigator to be sensory. In Auerbach's plexus these Type 11 cells are very prominent, and their several long dendrites are stated (though Cajal could not confirm this) to terminate upon the mucosa of the intestine. This would readily establish their sensory nature, but it does not seem possible to extend this idea to the cells of the sympathetic chain. At any rate, there is as yet no evidence to show that the numerous long, fine "dendrites" of these nerve cells emerge from the chain and end as sensory structures upon some viscus. Their nature seems a mystery, unless we put the obvious interpretation upon them, and regard them as fine neurones that have grown into the sympathetic cell. A comparison of figure 8 with figure 7 will make this more convincing.

The conclusion above reached, namely, that there are two distinct types of neurone junction in the sympathetic ganglia derives strong support from the observations of Dogiel upon the structure of Auerbach's plexus, and the nerve plexus in the gall bladder (11, 12). Two types of neurone junction certainly occur here. There are pericellular arborizations, enveloping the body of the cells, derived from fine medullated nerves, and therefore belonging apparently to the vagus nerve. But most of the fibres of the plexus are non-medullated nerves. These traverse the ganglia, giving off numerous collaterals, and eventually terminating in a distant ganglion. These collaterals[2] are described by Dogiel

[2] According to Dogiel the unmedullated fibres which traverse the plexus and give off collaterals in the various ganglia are in part sympathetic in origin, and in part arise from the motor cells of the plexus. He believes that the sympathetic fibres terminate on the cells of the plexus, and that such neurone relationship is not therefore confined to the vagal nerve fibres. On the other hand, Gaskell is of the opinion that the nerve cells of Auerbach's plexus are entirely vagal in origin, and that the vagal fibres alone form synapses with these cells. The sympathetic fibres, on the other hand, he believes, run an independent course to the musculature. The nerve cells of the plexus accordingly constitute the post-ganglionic portion of the vagus nerve, and the enteric system conforms, therefore, to the ordinary two-neurone (pre- and post- ganglionic) type, as found in the rest of the autonomic system. In favour of this is quoted the fact that nicotine abolishes the effects of vagal stimulation, leaving the effects of sympathetic stimulation unchanged (See W. H. Gaskell, "The Involuntary Nervous System"). This conclusion encounters the difficulty that the terminations of vagus fibres appear to be but few in number. Langley has noticed this same difficulty (see Langley, Journ. Physiol., vol. lvi, p. xxxix). He points out that while the vagal

as breaking up into the very finest threads, which ramify amongst the short dendrites of the motor cells of the ganglion (Dogiel type 1 cells). The precise relation of these very fine fibrils to the motor cells Dogiel could not determine; nevertheless, he believes that they merely come into contact with the dendrites of these cells.

The actual relation of these fine fibres to the motor cells is extremely difficult to observe, and this is due to the peculiarities of the methods of staining used. With the silver-chromate method Cajal is able to reveal with great clearness the myriads of fine fibrils that traverse the plexus, while at the same time the method leaves the motor cells of Dogiel unstained. It is obviously impossible under these circumstances to determine the relation of the fibrils to the motor cells.

The methylene blue process is usually equally indefinite. It has been my experience, and Dogiel's figure seem to indicate the same, that in preparations which clearly reveal the nerve cells of the ganglia, these nerve fibres are almost entirely unstained. The opposite also holds; and in preparations in which a thick feltwork of fine fibrils is strongly stained, nerve cells are recognizable only as clear, unstained spaces amidst the mass of fibres. Such preparations are again obviously unsuited for determining the relation of the fibrils to the nerve cells.

Nevertheless, if one examines a large series of ganglia, one occasionally finds cases where one or more nerve cells have been sharply stained, and where at the same time a small number of fibrils are rendered visible. It is these ganglia that throw most light upon the structure of the neurone junctions, and in these definite examples of neurone continuity may be detected. In figure 14 is shown a small motor cell from the myenteric plexus of the frog *(Hyla aurea)*. In a bundle of fine longitudinally running fibres (they have not been shown in the figure), one single fibre has stained sharply; it presents two varicosities, and gives off almost at right angles a very distinct collateral, which communicates directly with a small motor cell.

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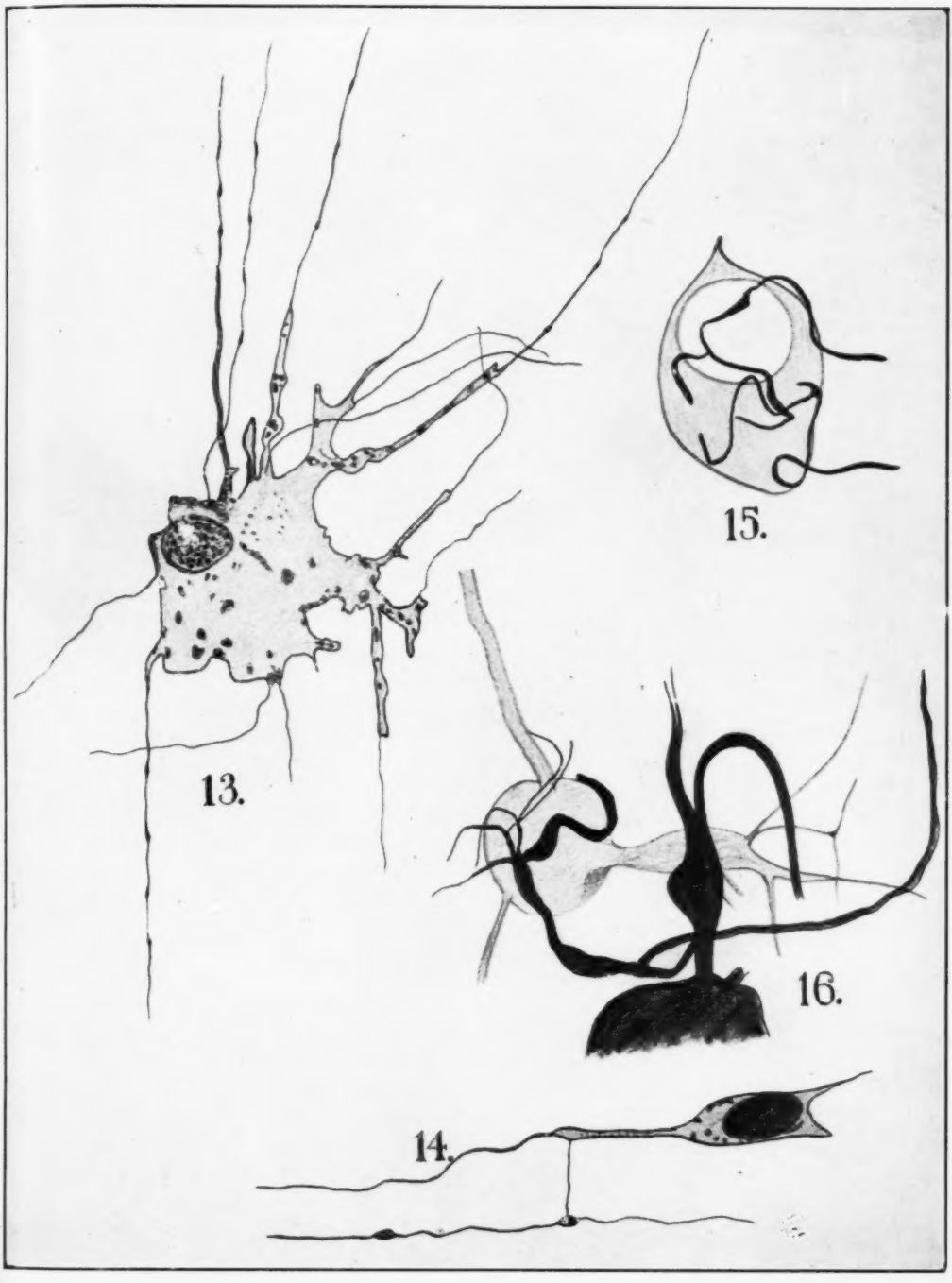
supply to the gut contains relatively few fibres, the cells of the plexus which this system supplies are to be counted probably in millions. He believes that the vagus fibres can terminate upon a relatively small number only of the cells of the plexus, and that the axons from these nerve cells branch extensively, and form junctions with the remaining cells of the plexus. This view is perhaps more in agreement with the findings of Dogiel, and is strongly supported by the studies of Erik Müller (Arch. f. mikr. Anat., 94 (1920), p. 208), on the development of the enteric nervous system in Squalus. On the other hand, it does not conform in plan with Gaskell's two-neurone system.

The view of Langley carries with it the possibility that the sympathetic nerve fibres might end upon the terminal cells of the vagal system, for we cannot now argue that nicotine would necessarily paralyze these cells in the manner in which it paralyzes ordinary post-ganglionic neurones. This might mean that the sympathetic and vagal nerve cells do not run an entirely independent course to the gut musculature, but that they converge upon a final common path; Dogiel's conclusions would then be correct. It does not seem, however, that pharmacological evidence can throw further light upon the question. Whatever the truth may be, it is fairly clear that the neurone terminations on the cells of Auerbach's plexus must have a double origin, one vagal (extrinsic), the other intrinsic; but whether the sympathetic fibres also form junctions with this system cannot yet be stated.


13.
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I find, moreover, that if the individual cells of the plexus are examined, fine fibrils may be seen apparently emerging from the dendrites. In the ganglion cells of the frog which I have examined, the great majority of the cells show such delicate short fibrils, apparently growing out of the dendrites. Sometimes they can be traced a considerable distance from the cell; at other times this is impossible, so faint and ill-defined do they become as we follow them away from the dendrites. What are these fine fibrils? That they are in direct connection with the dendrites cannot be doubted; figure 13 shows this with the greatest clearness. They are quite indistinguishable from nerve fibrils, and present the same fine varicosities as do these. Just as in figure 15 we see two pericellular expansions ending upon the body of a nerve cell, and at the same time showing no connection with the medullated fibre with which we know them to be connected, so it seems most obvious to conclude that these fine fibrils that enter the dendrites of the ganglion cells are the terminations of otherwise unstained non-medullated fibrils whose mode of ending Dogiel could not determine. There is, however, an objection that may be raised against this conclusion that we must carefully consider.

In Auerbach's plexus there are, as Dogiel has found, two types of cell (a third type which he has occasionally found does not concern us here). One of these, the type 1 (motor) cell, is characterized by the presence of short, slightly branched dendrites, and a single axon; the other, type 2 cell, is regarded as sensory. It is characterized by the presence of enormously long dendrites, indistinguishable from the axon in appearance, and terminating apparently in the epithelium of the intestine. The main point, then, by which we distinguish the two types of cell is that in the type 2 cell the short branching dendrites that characterize the type 1 cell are absent, so that the cell body has a relatively smooth contour, with a small number of extremely long, fine, varicose dendrites growing out from it. Nevertheless, Dogiel occasionally figures a cell, stated to belong to type 2, in which short dendrites occur, and from the ends of which the fine varicose fibres grow out.

Apart from such a fortunate case as that which I have shown in figure 14, where a fine branching fibre becomes continuous with a motor cell, the only other evidence which it is possible to get for a direct neurone continuity, is to observe fine fibrils, in the manner described above, apparently growing out from the nerve cells. Since in preparations where the nerve cells are clearly stained nerve fibres do not usually stain, it will be obviously impossible to trace these fine fibrils any great distance. Consequently, whenever such a cell is observed it will naturally be designated a type 2 cell, whose dendrites have incompletely stained, rather than a type 1 motor cell, into which fine fibrils (collaterals) from other cells have penetrated. Under these circumstances it is obviously impossible to obtain evidence for the growth of fibres into the dendrites of type 1 cells, since every time we find such a case we unhesitatingly call it a type 2 cell. But it is

very doubtful whether this procedure is justified. The type 1 cells are characterized by the presence of numerous short, branched dendrites, which are not typical of the type 2 cells. When we find cases of such cells, with fine fibrils apparently growing out of the dendrites, we seem much more justified in considering them as genuine cases of collaterals penetrating the type 1 nerve cells, than merely as abnormal cases of type 2 cells, with incompletely stained dendrites. In figure 13 is shown such a cell, into which at least fourteen fine fibrils seem to have penetrated. We are clearly not justified in calling this a type 2 cell, when in actual appearance it is indistinguishable from a type 1 cell (see, for example, figure 4b of Dogiel's paper).

The general conclusion which we draw from these observations is therefore as follows: *In the cells of the ganglia of the sympathetic trunk, and of Auerbach's plexus, two distinct types of neurone junction occur; fine collaterals penetrate the dendrites or the body of the nerve cells, and (this has been seen in the sympathetic ganglia only) become the neurofibrils of the nerve cells, undergoing anastomosis with the neurofibrils that have arisen in a similar way from other fine collaterals which have penetrated the cell. This anastomosing network is the seat of the integration. In addition to this type of neurone junction, which is the predominant one in the mammal, but does not occur in the sympathetic ganglia of the frog, there occur pericellular end-ramifications of nerves (seldom more than one on a cell), but it is not yet possible to say whether these end upon the surfaces of the cell or penetrate into its substance.*

Observations on Human Sympathetic Ganglia.

Special attention has been paid here to the structure of the neurone junctions in human sympathetic ganglia, since it is in this material that Cajal has made some of his most striking observations on neurone discontinuity at the synapse. Cajal's observations were made on the superior cervical ganglion.

In this ganglion Cajal distinguishes three main types of cell: (*a*) Cells with very long dendrites, (*b*) Cells with very short dendrites ending within the capsule, and (*c*) Cells having both types of dendrites. The last do not concern us here, since they merely combine the characters of the first two.

Concerning the first group, Cajal has observed that there are usually a very small number of neurones enveloping the body of the cells, whilst fibres may also be observed wound spirally round the dendrites. The spirally wound fibres, and the fibres which are wound round the body of the cells, constitute, he believes, the actual terminations of fibres upon this type of cell, i.e., the junction is believed to be one of contact and not of continuity. Of the dendrites it is stated that they divide occasionally, and eventually pass among bundles of fibres, often very far from their cell of origin, and eventually become lost. It is inferred, of course, that they eventually end blindly.

Concerning the second group, Cajal finds that the dendrites may be of two different kinds: thick, blunt dendrites, and very fine, delicate dendrites. The latter may break up into a bundle of very fine fibrils, which end generally close to their point of origin, just within the capsule that surrounds the cell. These cells are well supplied, at times extraordinarily so, with very delicate fibres which penetrate the capsule from without and ramify extensively among the thick and thin dendrites; again, the neurone junction is therefore believed to be one of approximation of neurones, rather than one of continuity. Cajal admits at the same time that the mass of fine fibres is so complex that it is impossible to unravel it and trace the fibrils individually.

My own observations have been made on human lumbar and coeliac ganglia, prepared by the reduced silver method. The former was from a man aged thirty years, the latter from an old man of seventy. Despite the fact that the ganglia were obtained more than eight hours after death, they gave excellent preparations, except that no indication of the internal neurofibril organization of the cells could be detected.

The three types of cell described by Cajal are readily detected, but the type with very short subcapsular dendrites is uncommon. In these ganglia I have not observed cells showing such extraordinary complexity as some of those figured by Cajal from the superior cervical ganglion. This really constitutes a considerable advantage, since in these cells of lesser complexity we have a much greater opportunity for following the individual fibres to their destination than in those complicated examples figured by Cajal, which, he himself states, he was unable to unravel.

I shall first describe the cells with dendrites which pass outside the cell capsule. In the lumbar and coeliac ganglia these constitute by far the majority of the cells. Sometimes the dendrites are quite delicate; at other times they are thick and massive, branch extensively, and become finer as we trace them a long way from the cell. Cajal has repeatedly stated that these various dendrites *terminate* sooner or later; my own observations show that this conclusion needs modifying. In the case of the fine dendrites, I find, as described by Cajal, that they usually break up into a few fine fibrils; but these fibrils, into which the dendrite has split, appear to terminate *because they pass out of the plane of the section*. A case of this is shown at X in figure 5. The short dendrite has split up into three fine fibres, but these only terminate because they emerge from the plane of the section; it is not, of course, possible to represent this on paper. When the large, thick dendrites are followed away from the cell, it is found that they undergo the same fate, but on a larger scale. They give off side-branches, which again branch in turn. These branches, which may be thick or thin (and eventually the dendrite itself also), after a longer or shorter distance, either become lost amongst other groups of fibres, or pass out of the plane of the section.

In such cases again it is impossible to detect any real termination of the "dendrite." When we observe the fine branches into which the dendrites have apparently divided, we observe in these a close resemblance to branches of axons. Those who maintain the theory of ramifying synapses ending upon nerve cells and their dendrites, will see in these fibres that have been given off from the dendrite merely an extensively branching dendrite system, and it is certainly difficult to disprove this assertion. There are, however, two objections which may be raised against it. Firstly, in the ganglia which I have examined most of the cells with long dendrites are quite devoid of fibres wound spirally round the dendrites, or terminating upon the body of the cell. It would be useless to object that they have merely not been rendered visible by the stain, for the staining was extraordinarily good. In this case we would have to conclude that these cells had practically no neurones ending upon them, which would be absurd. It seems much more probable that the fine fibres that appear to grow out from the dendrites are in reality branches of axons that have grown *into* the dendrites, in exactly the same manner as I have described in the cells of the spinal cord. It is possible to obtain more direct proof of this.

As I have pointed out in a previous paper (22), there are two means whereby we can recognize axon arborizations in sections of silver-nitrate material. The first is to trace these branches to the nerve cells from which they have originated, a method which is almost never applicable in practice. The second is to recognize the axon by the fact that it undergoes branching, there being usually a small triangular piece of protoplasm at the point where the branch is given off. The branch, even if it subsequently recurves, is always given off in the direction in which the axon is running, and it is in the more or less acute angle which this branch forms with the rest of the axon that the small triangular piece of protoplasm is situated.

The proof of this statement lies in the fact that if we observe a single well-defined axon in our preparations, its branches are all found to be given off in the one (forward) direction, and the triangular piece of protoplasm is always situated in the more acute angle which the branch forms with the axon.

While, therefore, the former method of detecting axons is of little value in this work, the latter can sometimes be usefully employed. Usually, it must be admitted, the branches which thus arise from the axons cannot be traced very far, for they soon leave the plane of the section, in the same way as do the branches which appear to grow out from the dendrites. But if very large numbers of such branches are patiently examined, sooner or later one is found which reaches its destination before it leaves the plane of the section. An excellent illustration of such a case is shown in figure 5 at Y, where the branch that arises from a neurone travels only a very small distance, and then grows into a large nerve cell. The neurone junction is obviously of a similar nature to that shown in figures 3 and 4, taken from the stellate ganglion of an unborn calf.

Now in figure 5 are shown a number of other fine fibres (dendrites we might call them), which after travelling a longer or shorter distance from the nerve cell pass out of the plane of the section. They have precisely the same appearance as the branching collateral Y. Doubtless, had the section been cut slightly differently, so as not to reveal the true nature of this latter fibre, it too would have been called a dendrite. It appears, therefore, that many of the very fine "dendrites" that seem to grow out from the cell are not dendrites, but rather branches from axons which have grown into the cell. At X, on the other hand, is shown a dendrite which breaks up into three fine branches, all of which emerge from the plane of the section. It is difficult to avoid the conclusion that these also are fine axon collaterals that have grown into the cell.

When we examine the fine branches which "grow out" from the large dendrites of the nerve cells, precisely the same observations may be made. Usually they leave the plane of the section, or become lost among a bundle of other similar fibres. But occasionally one is able to observe that such a branch, which seems to have grown out from the dendrite, is in reality the collateral from an axon, and therefore has evidently grown *into* the dendrite. The case is perhaps made most clear by reference to figure 11, which shows a thick but very short dendrite, obviously an outgrowth from the cell. From the ends of this dendrite there appear to grow out four fine fibres; they pass eventually out of the plane of the section, and we must look upon them, in reality, not as outgrowths from the dendrite, but as axon collaterals that have grown into the dendrite.

Similar remarks apply to the second type of cell seen by Cajal, those with dendrites ending within the cell capsule. These dendrites may be thick or very thin and delicate. My observations have been made only on the delicate ones. These may or may not break up into smaller branches, but in all cases when these branches are followed away from the cell they never end (as Cajal has maintained) beneath the capsule, but their ending is always a false one. Sometimes it is possible to trace them for but a short distance; at other times they may be traced a considerable distance within the capsule, but their ending is always an unreal one. Similarly, the fine fibres that penetrate from without into the capsule tend to become indistinguishable as individual fibres in the great mass of fibrils to which they give rise, and their terminations, whenever these occur, are always unreal. Cajal has himself admitted that it is practically impossible to follow individual fibres in this complex structure; how under such circumstances could a neurone discontinuity justly be maintained?

Reference must be made here to a particular type of dendrite relationship to which Cajal seems to attribute a physiological significance. In many sympathetic cells numerous small dendrites are given off from one pole only of the cell, and frequently two or three such cells may be contained within one capsule, with the dendrites mutually ramifying amongst one another. At other times the long dendrites from two cells may break up into small branches and ramify among

one another (for an illustration of this see Cajal's book, vol. ii, figure 56, at C and D). As this has been taken to represent a definite type of neurone connection, it may be pointed out that the figures are entirely misleading. The terminations of the dendrites are never real; if sufficient of them be contained in the section they are found to break up into fine fibres, for which no real termination can be detected. We must interpret such cases, therefore, not as definite neurone junctions; on the contrary, it would seem rather that the dendrites are approximated merely because they receive fibres from a common bundle of axon collaterals.

Physiological significance is also attributed by Cajal to cases of dendrites ramifying around the body of nerve cells. On the other hand, Kölliker and van Gehuchten were inclined to regard this as a mere accidental arrangement. I have drawn such a case in figure 16, in which it is seen that the short dendrites of one nerve cell break up into branches, which surround the body of another such cell. Cajal's statement that such dendrites actually terminate upon such a nerve cell seems to depend again upon his failure to realize that the endings which he observes are never anything but apparent endings. In the case shown in figure 16 the fine branches could be traced deeper and deeper down in the section, till they eventually emerged from it.

In human sympathetic ganglia one frequently observes fibres terminating in large, clear, structureless globules. The globules vary in size, but the largest may be nearly half the size of a ganglion cell. Cajal regards them as the termination of dendrites, and believes that they are pathological in nature, produced by the action of toxins and the senescent condition of the subject. I have been able to obtain some evidence in favour of this suggestion of Cajal. While in the ganglion cells of the thirty-year-old man they were entirely absent, they are found in extraordinary profusion in the ganglion of the old man of seventy. Some of them are quite small, others very large, and may often be observed close against a nerve cell, strongly indenting its walls. I have on several occasions observed large dendrites, otherwise normal in appearance, showing a pronounced swelling in one region. But these swellings are not confined to the dendrites, as Cajal believed, but may, on occasion, also be observed on the axons. A very clear case of this is shown in figure 6. One is very tempted in such preparations to suppose that these globules are the result of a gradual degeneration of the nervous elements of the senescent subject, and that such degeneration is manifested in a progressive breaking down of the neurone communications.

APPENDIX.

Note on Nerve Dendrites.

Some investigators have supposed that the dendrites are concerned in the process of nerve conduction; others maintain that they play no part in this, but that their rôle is a nutritional one. The conclusions above reached seem to throw some light upon their nature. The evidence leads us to conclude that axon collaterals, during embryonic life, grow into the body of the nerve cells, either singly or in bundles; the dendrite will then be that portion of the cell protoplasm which has grown outwards from the cell so as to envelop, or to collect within it, these fibrils that have grown into the cell. In reduced silver preparations, in which the dendrites as well as its neurofibrils have stained, this distinction between the dendrites which have grown out of the cell, and the fibrils that have grown into it, is easily observed, and in such preparations, as well as in most methylene blue preparations, the dendrites are found usually to be relatively short, only slightly branched structures. In the spinal cord they are seldom longer than several times the diameter of the cell from which they arise, though in the sympathetic ganglia they are frequently very much longer. But if the same cells are examined in Golgi preparations, they are found to be of enormous length, and in the case of the spinal cord may ramify over an extensive part of the section of the cord. The discrepancy is due to the fact that the Golgi method does not allow us to distinguish between the dendrites and the axon collaterals that have grown into it.

The above view is in harmony with the fact that those cells, such as the posterior root ganglion cells, upon which axon collaterals do not converge, are devoid of dendrites.

SUMMARY.

1. The investigation is an attempt to extend to the sympathetic system and its peripheral plexuses the observations previously made on the spinal cord, of a direct neurone continuity at the neurone junctions.

2. By the use of the methylene blue and reduced silver methods, using material from the cat, rabbit, guinea-pig, calf, frog, and man, it is observed that (except in the frog) the predominant type of ending is the same as that revealed in the spinal cord cells, i.e., the axon collaterals penetrate either into the body of the nerve cell, or more commonly into its dendrites. They pass as neurofibrils towards the centre of the cell, where they anastomose; this anastomosing network appears to be the seat of the integration.

3. In addition there occurs also a second type of ending, in the form of a pericellular ramification of nerve fibres, closely enveloping the body of the cell. This is the only kind found in the frog's sympathetic trunk. The end ramification lies beneath the cell capsule, but it is not possible to state whether it

penetrates into the protoplasm of the cell, or whether it ends in close contact with the cell surface. It is pointed out that a discontinuity of the staining reaction (as seen in Golgi and methylene blue preparations) constitutes no argument for discontinuity in the neurone junction; while, on the other hand, analogy with the motor ending of skeletal muscle is strong evidence in favour of protoplasmic continuity at the junction.

4. It is pointed out that, though the actual growth of axon collaterals into the dendrites constitutes the predominant type of neurone junction, it is only in rare cases that this may be *directly* observed.

5. Human sympathetic ganglia have been specially investigated, and it is concluded that the many remarkable cases of neurone discontinuity figured by Cajal are due to his failure to recognize the dendrite terminations as artefacts, due to their emergence from the plane of the section.

6. In the ganglia of a senescent, but not of a young human subject, the axons and dendrites may degenerate into large globules. Thus is brought about a gradual disruption of the nerve connections within the senescent ganglion.

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Figure 1. Large nerve cell from ganglion stellatum of kitten. Naked neurofibrils are to be seen "emerging from" various dendrites. In all cases the ending of the neurofibrils, and also of the other dendrites, is a false one, due to its leaving the plane of the section. It is, of course, not possible to represent this in the figure. Note that the neurofibrils run an independent course in the dendrites, but appear to undergo a limited amount of anastomosis within the body of the nerve cell.

(Cajal's reduced silver method.)

Figure 2. Nerve cells from thoracic sympathetic ganglion of kitten, amongst which a number of nerve fibres branch.

(Ehrlich's methylene blue injection method.)

Figure 3. From the thoracic sympathetic ganglion of unborn calf. One of the three nerve fibres is shown giving off a fine collateral, which comes into direct connection with a nerve cell.

(Cajal's reduced silver method.)

Figure 4. The same. One of the large nerve fibres is shown giving off a fine collateral, which fuses with one of the dendrites of the nerve cell. Several other fine fibres and three neuroglia nuclei are indicated.

(Cajal's reduced silver method.)

Figure 5. Sympathetic nerve cell with extracapsular dendrites. Two of the dendrites are thick and bulky, and are shown for only a small part of their length. At Y is shown a collateral from another axon, which gives off a moderately thick branch to the cell. No indication of discontinuity at the junction is observable. Two other similar fibres are shown at Z, apparently growing out from the cell (but in reality growing into it). Their relation to the other neurones cannot be demonstrated, since they emerge from the plane of the section. At X is shown a dendrite, into which three axon collaterals have penetrated.

(From Human Coeliac Ganglion of man aged 70 years; Cajal's reduced silver method.)

Figure 6. A nerve cell from coeliac ganglion of 70-years-old man. A large degeneration globule is forming on the axon. An indication of the cell capsule may be observed.

(Cajal's reduced silver method.)

Figure 7. Sympathetic cell from thoracic ganglion of kitten, prepared by Ehrlich's methylene blue injection technique. The axon, which gives off two collaterals, is indicated by a. Of the many fine processes which "grow out" from the cell, all have an unnatural ending, i.e., they may either emerge from the plane of the section, or, as frequently happens, the staining gradually fails. It is argued that these fine fibrils are really axon collaterals that have grown into the cell.

Figure 8. Sympathetic cell from thoracic ganglion of kitten, prepared by Cajal's reduced silver technique. Intracellular neurofibrils are impregnated, though far less satisfactorily than in spinal cord cells. The neurofibrils are observed to emerge from the cell and travel as naked neurofibrils in the ganglion. This cell is evidently of a similar kind to that depicted in figure 7. The distinction between extracellular fibrils and dendrites is much more obvious than in figure 7, and the figure illustrates with greater force the thesis maintained in this paper, that the fine fibrils are not dendritic *outgrowths* from the cells, but axon collaterals that have *entered the cell.*

Figures 9 and 10. The same. The cell capsule is clearly seen in these two cells.

Figure 11. Cell from coeliac ganglion of old man. The distinction between dendrites and the fibrils that have grown into them is obvious.

(Cajal's reduced silver method.)

Figure 12. Three nerve cells from sympathetic ganglion of frog, with pericellular nerve termination. In two of the cells the spiral fibre breaks up into finer fibrils on the surface of the cell. In the other a fibre is wound round and round the nerve cell.

(Cajal's reduced silver method.)

Figure 13. A "motor" cell (Dogiel type 1 cell) from Auerbach's plexus. It is maintained that the fine fibrils that appear to grow out from the dendrites are axon collaterals that have grown into the cell. (Duodenum of rabbit; methylene blue.)

Figure 14. A "motor" cell from Auerbach's plexus, receiving a fine collateral from a non-medullated nerve fibre. (Frog [*Hyla aurea*]; methylene blue.)

Figure 15. A cell from Auerbach's plexus, with two pericellular nerve-endings. (Rabbit; methylene blue.)

Figure 16. Two nerve cells from human coeliac ganglion. One of the nerve cells (part of which only is shown), gives off a dendrite whose branches surround the body of the second cell. It is observed, however, that these branches are produced into fine fibrils, and that these fibrils end merely because they have been cut short. It is maintained that this does not constitute a termination of the dendrites upon the cell; on the contrary, the fine fibrils are axon collaterals that have entered the dendrites, and the peculiar disposition of the latter around the body of the nerve cell is purely accidental.

(Cajal's reduced silver method.)

WHETHAM'S LAW AND THE LAW OF MASS ACTION; AND THEIR APPLICATION TO THE COAGULATION OF COLLOIDAL PLATINUM

by

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Whetham (13) has generalized the coagulating effect of ions on colloids carrying the opposite charge in the following words: "The coagulating power of a series of ions of the same sign is proportional to a constant raised to the power representing the valence of each ion." This law at the time of its enunciation received quite startling confirmation from the meagre figures at hand, and hence was widely accepted.

Whetham deduced this generalization by the method of probability, but Robertson (11) points out that the same result is obtained by the application of simple mass action principles, such principles being based on the theory of probability.

It is shown in this paper that Whetham's law, and also the law of mass action when based on principles corresponding to those on which Whetham's law is based, lead to quantitative relations which are not supported by experimental facts; and it is concluded that Whetham's law is theoretically unsound, and also that the fundamental assumptions inherent in this particular application of mass law principles are incorrect.

The Application of the Mass Law to the Coagulation of Colloids by Electrolytes.

It will be convenient for the sake of a clear theoretical presentation to discuss the mass law application first, and to divide such discussion into two parts.

(A). The colloid to be precipitated invariably carries an electric charge, and it is assumed that coagulation occurs when the charge is wholly or partially neutralized by combination with an ion carrying the opposite charge.

Suppose that each colloid particle combines with $6n$ unit charges before it is brought into the coagulable state, and for convenience suppose that it is thereby

neutralized. Let the concentration of the colloid to be precipitated be Q, and let the concentrations of the uni-, bi-, and tri-valent ions necessary to produce coagulation in equal times be C_1, C_2, and C_3, in gramme ions per litre. As these concentrations are invariably low, C_1, C_2, and C_3 may also be taken as representing the respective activities.

The reactions for univalent, bivalent, and trivalent salts are then given by the following equations:

$$\left.\begin{array}{ll} \text{(1a)}. & 6n\ Na^{+} + \text{1 colloid particle} \xrightarrow{(6n-)} \text{Colloid} \cdot (Na)_{6n} \\ \text{(1b)}. & 3n\ Ca^{++} + \text{1 colloid particle} \xrightarrow{(6n-)} \text{Colloid} \cdot (Ca)_{3n} \\ \text{(1c)}. & 2n\ Al^{+++} + \text{1 colloid particle} \xrightarrow{(6n-)} \text{Colloid} \cdot (Al)_{2n} \end{array}\right\} (1)$$

Now n can be integral or fractional, and in either case the following holds. Calling the initial velocities of the respective reactions V_1, V_2, V_3, and applying the mass law, one obtains:

$$\text{(1d)}.\quad V_1 = k_1 \cdot Q \cdot C_1^{6n}$$
$$\text{(1e)}.\quad V_2 = k_2 \cdot Q \cdot C_2^{3n} \text{ and}$$
$$\text{(1f)}.\quad V_3 = k_3 \cdot Q \cdot C_3^{2n}$$

where K_1, K_2, and K_3 are the respective velocity constants. Now, it is the practice in coagulation experiments to mix the colloid and electrolyte, to hold for some stated time, say, three minutes, ten minutes, or even twenty-four hours, and then to examine the mixture to see if there be any evidence of partial or total coagulation. The smallest concentration of electrolyte which gives definite evidence of coagulation at the end of this fixed time is taken as "the coagulation concentration." It is evident then that this, the usual method, directly compares the coagulation velocities of the reactions concerned. The necessary relations for equal coagulative velocities in the foregoing equations are given by

$$k_1 \cdot Q \cdot C_1^{6n} = k_2 \cdot Q \cdot C_2^{3n} = k_3 \cdot Q \cdot C_3^{2n},$$
$$\text{or } k_1 C_1^{6n} = k_2 C_2^{3n} = k_3 C_3^{2n},$$

which is therefore the relation between the concentrations of uni-, bi, and trivalent ions necessary to coagulate a given colloid in a given time.

No simpler relation follows unless we make the simplifying assumption that the three velocity constants are equal, that is, that the three reactions in equation (1) are not chemically specific, but are purely electrical in nature. It must be stressed that this rather sweeping assumption is essential for the carrying further of the general deduction.

With such an assumption we have at once:

$$\text{(1g)}.\quad C_1^{6n} = C_2^{3n} = C_3^{2n}.$$

Utilizing these relations,

$$(C_1^{6n} = C_2^{3n} \text{ and } C_1^{6n} = C_2^{2n})$$

and taking the $3n$-th root and the $2n$-th root respectively, we get,

$$\text{(1h)}.\quad C_2 = C_1^{2} \text{ and } C_3 = C_1^{3}.$$

We may then write:

$$C_1 : C_2 : C_3 = C_1 : C_1^2 : C_1^3$$
$$\text{or} \quad 1 : C_1 : C_1^2.$$

Using p for the coagulating power (the reciprocal of the coagulating concentration), it follows that

$$(1k). \quad p_1 : p_2 : p_3 = 1 : \frac{1}{C} : \left(\frac{1}{C_1}\right)^2$$

or in the more usual form:

$$1 : X : X^2.$$

Thus the general mass law deduction leads to the usual form of Whetham's law, but with this important difference, the constant X is not an arbitrary constant, but is given by $\frac{1}{C}$. Further, two interesting relations revealed by this deduction are that $C_2 = C_1^2$ and $C_3 = C_1^3$, or the coagulating concentration of the bivalent ion is the square of the coagulating concentration of the univalent ion, and that of the trivalent ion is the cube of the coagulating concentration of the univalent ion.

An interesting point arises when we consider the case where n in equation (1) above is fractional. For instance, if n equals $\frac{1}{2}$ the equations in (1) become:

$$\left.\begin{array}{ll} (2a). & 3Na^+ + 1 \text{ colloid particle } \overset{(3-)}{\longrightarrow} \text{Colloid . } (Na)_3 \\ (2b). & \frac{3}{2}\, Ca^{++} + 1 \text{ colloid particle } \overset{(3-)}{\longrightarrow} \text{Colloid . } (Ca)_{3/2} \\ (2c). & 1Al^{+++} + 1 \text{ colloid particle } \overset{(3-)}{\longrightarrow} \text{Colloid . } (Al) \end{array}\right\} (2)$$

The foregoing general deduction holds if the equations are left in this form; but equation (2b) is an unusual form for the application of the mass law, and if it be thrown into the more usual form,

$$3Ca^{++} + 2 \text{ Colloid particles } \overset{(3-)}{\longrightarrow} (\text{Colloid})_2 \text{ . } Ca_3,$$

the value of the velocity constant k_2 becomes altered accordingly. As the general deduction requires the assumption that the three constants, k_1, k_2, and k_3, are equal, it is evident that one cannot be altered unless the other two are correspondingly altered. This then raises the question as to what are really the true comparable settings (if any) for the three equations. A little consideration shows that the most probable settings are those that represent that the same quantity of colloid is coagulated, for experimental measurements are made when the same approximate quantity of coagulated colloid comes into view (or is completely thrown down). If then one of the above equations is multiplied by some factor, e.g., 2, all three equations must be multiplied by this factor. The three velocity constants then become altered to the same degree, and the equations still represent that the same quantities of colloid are coagulated. Under such conditions the relations due to the foregoing deduction will always theoretically follow.

If, however, different factors are introduced into the three equations, then

the foregoing relations between the precipitating powers will no longer hold, but quite arbitrary relations, depending on the factors introduced, will be obtained.

(B). Robertson (11) did not use the above settings for the equations, but assuming that one colloid particle combined with one univalent ion, and two colloid particles with one bivalent ion, etc., he wrote the equations:

$$\left.\begin{array}{ll} \text{(3a).} & 1Na^{+} + 1 \text{ colloid particle} \longrightarrow \text{Colloid} \,.\, Na \\ \text{(3b).} & 1Ca^{++} + 2 \text{ colloid particles} \longrightarrow (\text{Colloid})_2 \,.\, Ca \\ \text{(3c).} & 1Al^{+++} + 3 \text{ colloid particles} \longrightarrow (\text{Colloid})_3 \,.\, Al \end{array}\right\} \quad (3)$$

This is equivalent to writing n equal to 1/6 in the general equation (1), and then multiplying (1b) by 2, and (1c) by 3. The introduction of these different factors into the equation should, according to the foregoing, give rise to a different proportionality between the coagulating powers from the general one deduced:

$$\left[p_1 : p_2 : p_3 = 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2\right]$$

This is so; for equating the velocities in the actions represented by equation (3) we have:

$$\text{Velocity} = k_1 \,.\, Q \,.\, C_1 = k_2 \,.\, Q^2\, C_2 = k_3 \,.\, Q^3 \,.\, C_3.$$

From which

$$C_1 : C_2 : C_3 = \frac{1}{Q} : \frac{1}{Q^2} : \frac{1}{Q^3},$$

$$\text{or } p_1 : p_2 : p_3 = 1 : Q : Q^2$$

where Q is the colloid concentration.

Equations (3a), (3b), and (3c), however, represent particular cases of the general equations.

$$\left.\begin{array}{ll} \text{(4a).} & n.Na^{+} + 1 \text{ colloid particle} \overset{(n-)}{\longrightarrow} \text{Colloid} \,.\, (Na)_n \\ \text{(4b).} & n.Ca^{++} + 2 \text{ colloid particles} \overset{(n-)}{\longrightarrow} (\text{Colloid})_2 \,.\, (Ca)_n \\ \text{(4c).} & n.Al^{+++} + 3 \text{ colloid particles} \overset{(n-)}{\longrightarrow} \text{Colloid})_3 \,.\, (Al)_n \end{array}\right\} \quad (4)$$

On equating velocities and proceeding as above we obtain:

$$\text{(4d).} \quad p_1 : p_2 : p_3 = 1 : Q^{1/n} : (Q^{1/n})^2.$$

It is at once noticed that the proportionality between the coagulating powers is in the same form as the previous deduction, namely: $1 : X : X^2$, but whereas X had the value $\frac{1}{C_1}$ it has by this deduction the value $Q^{1/n}$ where Q is the colloid concentration.

Deduction of Whetham's Law.

Whetham's law itself is deduced on principles which are comparable at every step with those used in the foregoing deduction (A), and further it is based on practically the same assumptions.

These assumptions are:

(*a*) That the charged colloid is coagulated when its charge is neutralized by a certain minimum ionic charge.

(*b*) That the rate of neutralization, or the probability of the electrical conjugations, is proportional to the concentration of the ions.

(*c*) That the conjugations are electrical only, and are not chemically specific.

Whetham proceeds essentially as follows:

The necessary coagulations occur when $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, meet 1 colloid particle. (Compare with equations 1a, 1b, and 1c). "The probability of an ion being within reach of a fixed point at any instant is, putting certainty equal to unity, approximately represented by a fraction proportional to the ratio between the volume occupied by the sphere of influence of the ions and the whole volume of the solution, and may be written as $A \cdot C^1$, where A is a constant and C^1 represents the concentration of the solution," i.e.:

$$(5). \quad \text{Probability} \propto A \cdot C^1.$$

(We shall write C^1 for any arbitrary concentration and C for the coagulation concentration.)

The probability of the conjugation of n ions at the one fixed point is then given by $(AC^1)^n$. For the same frequency of conjugation of $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, we should have:

$$(AC_1)^{6n} = (AC_2)^{3n} = (AC_3)^{2n} = \text{constant} = B,$$

where C_1, C_2, and C_3 represent the respective coagulation concentrations.

It is evident then that

$$C_1 = \frac{B^{1/6n}}{A}$$

$$C_2 = \frac{B^{1/3n}}{A}$$

$$C_3 = \frac{B^{1/2n}}{A}$$

From these relations it follows that:

$$(6a). \quad (AC_1)^2 = AC_2, \text{ or } \frac{C_2}{C_1^2} = A, \text{ and}$$

$$(6b). \quad (AC_1)^3 = AC_3, \text{ or } \frac{C_3}{C_1^3} = A^2, \text{ and}$$

$$(6c). \quad p_1 : p_2 : p_3 = 1 : \frac{1}{AC_1} : \frac{1}{AC_2}$$

$$\text{or } 1 : \frac{1}{AC_1} : \left(\frac{1}{AC_1}\right)^2$$

The last relation is in the form $1 : X : X^2$, where $X = \frac{1}{AC_1}$. In the original deduction Whetham does not bring out this inter-relation $\left(X = \frac{1}{AC_1}\right)$ but takes X to be an arbitrary constant. X, however, is known if A can be found, and the latter can be obtained from (5) as follows:

$$AC = \frac{\text{Volume of the spheres of influence of the ions}}{\text{Total volume}}$$

$$= \frac{V}{\text{Total volume}}$$

$$\text{Therefore } A = \frac{V}{\text{Total volume}} \times \frac{1}{C}$$

$$= \frac{V}{\text{Total volume}} \times \frac{\text{Total volume}}{\text{No. of gramme ions}}$$

$$= \frac{V}{\text{No. of gramme ions}}$$

Hence A is the volume of the sphere of influence of 1 gramme ion (in litres).

This will be referred to later in dealing with the experimental figures.

There is, however, one interesting point that has so far been overlooked. The concentrations C_1, C_2, and C_3 are the coagulation concentrations, and we may take them theoretically and experimentally as representing the concentrations at which coagulation is instantaneous; in which case the probability of the conjugations occurring becomes certainty, and hence AC_1 (where C_1 is the coagulation concentration) becomes equal to 1. The proportionality $p_1 : p_2 : p_3$ being equal to $1 : \frac{1}{AC_1} : \frac{1}{(AC_1)^2}$, then assumes the anomalous form $1 : 1 : 1$.

It may be objected that there is no method of detecting instantaneous coagulation, and that there must be a time limit, however small. This is true, and, in fact, there should be a time factor in all probability deductions, owing to the probability of an event occurring being some function of the time. Further, in this particular probability application a time factor is necessary, as the coagulation concentrations are dependent on (and vary with) the time interval over which observations are taken. If a time factor be introduced, and if we then write, "The probability of an ion being within reach of a fixed point," *within a certain time interval*, say, 1 second, or 1 minute, or 24 hours, is proportional to A . C, we obtain by following the usual deduction exactly the same relation:

$$p_1 : p_2 : p_3 = 1 : \frac{1}{AC_1} : \frac{1}{AC_2}$$

Also, as C_1 (and C_2 and C_3) are the concentrations which cause coagulation within that fixed time limit, we obtain in the same way the same anomalous results:

$$p_1 : p_2 : p_3 = 1 : 1 : 1.$$

It therefore appears to the author that the theory of probability can be applied only with certain limitations and only in the following way.

The probability of an ion being within reach of a fixed point within a certain time interval is proportional to the number of such ions, and also to the number of such fixed points, the latter representing (theoretically) the colloid particles. The probability of such a meeting is therefore proportional to the product of the ionic concentration (C) and the colloid concentration (Q). The probability of the simultaneous conjugation (within the fixed time limit) of 6 ions and any 1 colloid particle is then proportional to C^6Q. For the same frequency of conjugation of $6n$ univalent ions, or $3n$ bivalent ions, or $2n$ trivalent ions, we therefore have

$$(7). \quad Q.\,C_1{}^{6n} = Q.\,C_2{}^{3n} = Q.\,C_3{}^{2n} \text{ and}$$
$$C_1{}^{6n} = C_2{}^{3n} = C_3{}^{2n}$$

which is exactly the relation deduced from mass law principles (see equation 1g). That this should be so is evident when we consider, (*a*) that the mass law deductions are based directly on probability principles, (*b*) that in the above case we are concerned with the same fundamental reactions, namely, the conjugation of $6n$ univalent, $3n$ bivalent, and $2n$ trivalent ions with 1 colloid particle, and (*c*) that the frequency of such conjugations directly controls the velocity of the reaction. We have every evidence that the mass law is fundamentally sound, and it is questionable whether probability deductions will give us any more information than the simple application of the mass law. In this particular case the author considers that they do not, but that both lead to the same results as represented by equations (7) and (1g). On treating equation (7) in the same way as (1g) we obtain:

$$(8). \quad p_1 : p_2 : p_3 = 1 : \frac{1}{C_1} : \frac{1}{C_2}$$
$$= 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2$$
$$= 1 : X : X^2$$

EXPERIMENTAL PART.

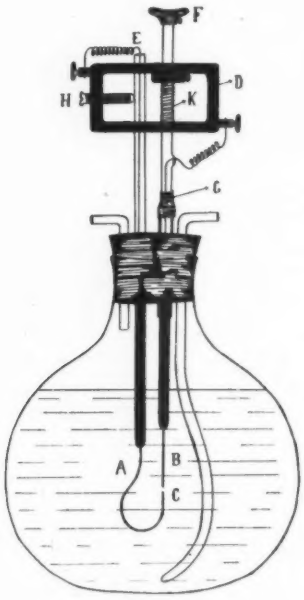
A study of the literature shows varying coagulation figures for the same colloid, owing chiefly to differences in the methods of preparation of the colloid, and to its degree of purity or freedom from traces of stabilisers.

Varying figures are also obtained, depending on the time interval before observation and on the method of adding the electrolyte (4, 7, 9, 10). Although the great majority of these figures could be used (and have been used) to show the general weakness of Whetham's law, it was considered advisable, in order to test the points raised in the foregoing discussion, to procure a set of experimental figures for a colloid which was quite free from traces of stabilisers and other

impurities. To this end very pure samples of colloidal platinum were prepared, particularly free from foreign electrolytes.

Preparation of Colloidal Platinum.

The platinum sols were made in conductivity water by the Bredig method. For the preparation of the conductivity water a still after the design of Bourdillon (1) was employed, except that the condenser, with its three cooling jackets,



was made of boro-silicate glass and all in one piece, having an over-all length of 36 inches. All the glass vessels used, including the tubes in which the coagulations were carried out, were of boro-silicate glass. The conductivity water was siphoned from the collecting vessel into the sparking vessel, always in the absence of CO_2, and immediately before sparking a sample was siphoned over and its conductivity measured. The specific conductivity of the water used in the four separate preparations lay between ·3 and ·5 gemmho. The sparking was carried out with pure platinum poles in the apparatus shown in the figure. The platinum electrodes A and B were sealed into two Pyrex tubes, which passed through a rubber cork. The tube AE fitted firmly into the cork, whilst the tube BF passed through a short but wider tube in the cork. A small piece of rubber tubing at C

supported the tube BF, and at the same time allowed the slight freedom of movement necessary to close the spark gap C. The cork fitted firmly into the mouth of a short-necked 500 cc. Pyrex flask. Both tubes also passed loosely through the hollow ebonite block D. The tube AE was held securely by the screw H, whilst the tube BF had the small spiral spring K attached to it. On compressing F the spark gap C could be closed, and on slightly releasing F the spring K would open C to any desired extent. Inlet and outlet tubes were provided as shown, so that CO_2 free air could be steadily bubbled through the solution during the process of preparation. These tubes also served for the introduction of the conductivity water and for the removal of the prepared sol. The solutions were always prepared in exactly the same way, and as follows: The apparatus was carefully cleaned and the cork put into position. About 450 cc. of conductivity water was then siphoned in, and a sample siphoned into the conductivity cell, and its conductivity measured. The sparking vessel was then placed in a freezing mixture (about —4°), and by manipulating F the sparking was carried on intermittently for about one half-hour with a current of about 6 amps. During the whole process the vessel was gently shaken and the solution kept as near 0° as possible. To this end no arc was held for more than about 10 seconds. After the preparation the sol was siphoned into another flask, and then boiled. It was then set aside for 24 hours, when the pure sol was carefully siphoned over from the precipitate. Generally two successive lots of about 400 cc. each were prepared as above, and then mixed. For the purpose of the experiment it was necessary to be able to prepare comparable samples of the Pt. sol. This was achieved by following the same procedure in each preparation, but making the sols a little stronger than necessary, and then diluting with conductivity water till the boiled sols showed the same specific conductivity. The specific conductivity of the standard sol used in these experiments was 12·5 gemmhos.

The Coagulation Procedure.

The coagulation concentration varies with the time and with the temperature. In the preliminary experiments the observations were taken three minutes after mixing, and in a glass-fronted water bath at 30°, using a magnifying eye-piece, and utilizing as a background a highly illuminated glazed white tile. The black coagulated particles showed up well. This method, however, did not give a sufficiently sharp coagulation concentration, and it was finally utilized only for quick and approximate indications of the necessary concentration of electrolyte required. The method that gave the best results, and which was used exclusively for the results set out in Tables 1 and 2, consisted of mixing the solutions at the room temperature, shaking by hand for one minute, and then putting in the water bath at 30° for 24 hours. It was found that 24 hours served as a convenient minimum time interval, sols uncoagulate after that time remaining stable for at

least several days. If necessary, observations were taken with the white background, but generally the time interval of 24 hours was sufficient to allow the coagulated particles to settle completely, and therefore observations became simple.

The coagulation concentration of electrolyte was obtained in the following way: Preliminary experiments having given a fairly close approximation of the required concentration of any particular electrolyte, a range of 6–10 solutions was prepared having electrolyte concentrations varying round this approximate figure. These solutions generally differed from one another by a maximum of 10%, so that the coagulation concentrations obtained in this work can only be considered accurate to within 10%. This is quite sufficient for the purpose in hand, and, in fact, quite apart from the tedious work involved when a considerable number of electrolytes have to be investigated, it is sometimes very difficult to obtain a coagulation concentration within narrower limits. Each of the above solutions was prepared by putting the necessary quantity of electrolyte into a steamed and air-dried Pyrex test tube, adding sufficient water to make ·3 cc., and then rapidly running in 2·5 cc. of Pt. sol. It was necessary to use micropipettes; these were carefully checked, and the percentage error associated with the technique employed was determined. The concentration of the added electrolyte was then so arranged that the percentage error due to the use of these small quantities was always less than 2%. In view of the foregoing, this degree of accuracy was quite satisfactory.

TABLE 1.

Coagulation Concentrations are given in Gramme Ions of Cation per litre, assuming the activity coefficient of the cation to be unity.

	Mean coag. conc.	Mean coag. power.	Relative coag. power.
Univalent Cations.			
Na Acetate	·010	100	1
$\frac{1}{2}K_2Cr\ O_4$	·0095	105	1·05
KNO_3	·0081	123	1·2
KCN	·0058	172	1.72
NaCl	·0048	208	2·1
KCl	·0036	278	2·8
$RbNo_3$	·0022	455	4·5
$CaNo_3$	·0022	455	4·5
NH_4NO_3	·0021	476	4·8
NH_4Cl	·0015	667	6.7
$\frac{1}{2}K_2Cr_2O_7$	·0014	714	7·1
$AgNO_3$	·00032	3,120	31

	Mean coag. conc.	Mean coag. power.	Relative coag. power.
Bivalent Cations.			
$CaCl_2$	·00013	7,690	77
$Ca(NO_3)_2$	·00012	8,330	83
$BaCl_2$	·000072	13,900	139
$Zn Cl_2$	·000062	16,100	161
$CuSO_4$	·000026	38,500	385
$CuCl_2$	·000018	56,000	560
Trivalent Cations.			
$FeCl_3$	·0078	128	1·28
$\frac{1}{2}Fe_2(SO_4)_3$	·000040	25,000	250
$LaCl_3$	·000011	90,900	909
$\frac{1}{2}Al_2(SO_4)_3$	·0000087	115,000	1,150
$AlCl_3$	·0000080	125,000	1,250
$Y(NO_3)_3$	·0000079	127,000	1,270
$\frac{1}{2}Di_2(SO_4)_3$	·0000067	150,000	1,500
$Ce(NO_3)_3$	·0000066	152,000	1,520

Note on Table I.

The mean coagulation concentrations only are given, and represent the mid-values between pairs of concentrations, one of which is just insufficient to produce coagulation and the other just able to bring it about. As has been pointed out, these mean values are liable in general to a maximum error of 10 per cent.

TABLE 2.

Effect of Altering the Colloid Concentration.

	1		2		3	
	a Coag. conc. of electro-lyte.	b Relative coag. power.	a Coag. conc. of electro-lyte.	b Relative coag. power.	a Coag. conc. of electro-lyte.	b Relative coag. power.
Univalent Cations.						
$NaCl$	·0048	2·1	·0061	1·6	·0079	1·3
KCl	·0036	2·8	·0047	2·1	·0062	1·6
$RbNO_3$	·0022	4·5	·0031	3·2	·0037	2·7
$CsNO_3$	·0022	4·5	·0026	3·8	·0032	3·1
NH_4Cl	·0015	6·7	·0015	6·7	·0015	6·7
$AgNO_3$	·00032	31·3	·00032	31·3	·00034	29·4

	1		2		3	
	a Coag. conc. of electro-lyte.	b Relative coag. power.	a Coag. conc. of electro-lyte.	b Relative coag. power.	a Coag. conc. of electro-lyte.	b Relative coag. power.
Bivalent Cations.						
$Ca(NO_3)_2$	·00012	83	·00012	83	·00012	83
$BaCl^2$	·000072	140	·000075	133	·000075	133
$CuSO_4$	·000026	385	·000026	385	·000024	417
Trivalent Cations.						
$Al_2(SO_4)_3$	·0000089	1,120	·0000078	1,280	·0000062	1,610
$AlCl_3$	·0000080	1,250	·0000069	1,450	·0000060	1,670
$Y(NO_3)_3$	·0000081	1,230	·0000067	1,480	·0000052	1,920
$Ce(NO_3)_3$	·0000063	1,590	·0000050	2,000	·0000050	2,000

Column 1 gives the coagulation concentrations and relative coagulating powers of the various electrolytes using the standard Pt. sol.

Column 2 gives the corresponding figures when the Pt. sol has been diluted to ·6 of its original strength.

Column 3 gives the corresponding figures when the Pt. sol has been diluted to ·4 of its original strength.

An examination of Table 1 shows large variations amongst the coagulating concentrations of cations of the same valence. In general, similar variations are shown with most colloids (3, 5, 6, 7, 12), and it is sometimes concluded that these variations alone are sufficient to condemn Whetham's law. However, the wide differences in coagulating powers of uni-, bi-, and trivalent ions far outshadow these lesser variations, and the Hardy-Schultz qualitative law stands out. It is instructive, therefore, to neglect the more exceptional cases in the table (e.g., $AgNO_3$ and $FeCl_3$), and to examine the foregoing deductions in terms of a standard representative of each of the valence series. To this end we shall take KCl as representative of the univalent series, $BaCl_2$ of the bivalent, and $AlCl_3$ of the trivalent. As these have the same anion (namely, chlorion), the comparison is apt. It is, of course, recognized that the general conclusions must be very approximate, but as will be seen, the theoretical and practical results are not only far from approximate agreement, but they vary so enormously as to demand the conclusion that the theoretical basis is unsatisfactory.

The foregoing mass law deduction (A), and also Whetham's law when deduced on comparable lines, lead to the proportionality:

$$p_1 : p_2 : p_3 = 1 : \frac{1}{C_1} : \frac{1}{C_2}$$

$$\text{or } 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2 \quad \text{(equations 1k and 8).}$$

The coagulating concentrations C_1, C_2, and C_3 of KCl, $BaCl_2$, and $AlCl_3$ are respectively ·0036, ·000072, and ·000008, and the respective coagulating powers 2·8, 139, and 1,250 (Table 1). The proportionality experimentally obtained is therefore

$$(9a). \quad p_1 : p_2 : p_3 = 1 : 50 : 450.$$

The proportionality using the above theoretical relations is, however,

$$(9b). \quad 1 : 278 : 14{,}000 \left(\text{Using } 1 : \frac{1}{C_1} : \frac{1}{C_2}\right)$$

or

$$(9c). \quad 1 : 278 : 77{,}000 \left[\text{Using } 1 : \frac{1}{C_1} : \left(\frac{1}{C_1}\right)^2\right]$$

The experimental relation (9a) differs so seriously from the theoretical (9b) and (9c) that we must conclude that the theoretical proportionality does not give any quantitative indication of the relative coagulating powers.

Whetham's constant A has been shown to be the volume of the sphere of influence of a gramme ion, and in the ordinary deduction is taken as constant for all ions. If C_1, C_2, and C_3 represent the coagulation concentrations, then, as the probability of the necessary conjugations occurring is now certainty, the product of the volume of the sphere of influence of each ion multiplied by its concentration must in each case become equal to unity. Hence AC_1, AC_2, and AC_3, for the salts KCl, $BaCl_2$, and $AlCl_3$, are all equal to 1, and therefore A, which is assumed to be constant, varies from 278 to 14,000 to 125,000.

In the foregoing deductions it is assumed that in all cases the given colloid is being used at constant concentration, and therefore in the deduced proportionalities the term expressing this concentration does not appear. It is evident, however, that the probability of the occurrence of the necessary conjugations or the velocity of coagulation must be directly proportional to Q, the colloid concentration. This means that the coagulating power of any cation should be proportional to Q. Table 2, which gives the coagulating concentrations and the coagulating powers at varying colloid strengths, shows that this is by no means the case. With univalent cations the coagulating power does decrease in general with decrease in colloid concentration, but a direct proportionality does not exist. With bivalent cations the coagulating power seems to be definitely independent of colloid concentration, and with trivalent cations the coagulating power actually increases with decrease in colloid concentration. Similar results are obtained by Kruyt and van der Spek (8), using other colloids. It is evident that such variations must cause different coagulation proportionalities to be obtained according to the strength of colloid used. Using KCL, $BaCl_2$, and $AlCl_3$ from Table 2, the coagulating powers of the standard platinum sol are 1 : 50 : 450, whilst the corresponding powers when the sol is diluted to ·4 of its original strength are 1 : 83 : 1,040. Burton and Bishop (2) using arsenious sulphide sol over a much wider range of dilution, show a correspondingly wider range of

variation in coagulating powers. Such results are obviously in direct contradiction to the theoretical requirements.

Deduction (B) in the foregoing theoretical treatment leads to relation (4d), namely, $p_1 : p_2 : p_3 = 1 : Q^{1/n} : (Q^{1/n})^2$. The actual value of n is unknown, but from equation (4a) it is at once evident that n must always be integral and greater than 1. Further, the colloid concentration, Q, is generally only approximately known; but the colloid solution can always be diluted until Q is less than unity. When such is the case, $Q^{1/n}$ is obviously less than unity; and the relation $p_1 : p_2 : p_3$ becomes a decreasing proportionality. This makes the univalent ions the strongest coagulating agents and the trivalent the weakest. As this is in direct contradition to all experimental results, deduction (B) must be inherently incorrect.

In conclusion, it should be mentioned that solutions of colloidal platinum have been found by the author to be definitely acid. The standard sols used in this work (specific cond. 12·5 gemmhos) showed by direct conductivity, and also by conductometric titration, an acid normality of ·00004. The evidence definitely indicates a surface ionization, and in a later paper the author will attempt to show that the coagulation of colloidal Pt. is due to a surface salt formation.

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INFECTIOUS NECROTIC HEPATITIS OF SHEEP IN VICTORIA. A BRAXY-LIKE SHEEP DISEASE

by

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During an investigation into braxy-like diseases of sheep in Victoria, the author encountered a condition which bore marked resemblances to Black Disease (Dodd), and for which the name Infectious Necrotic Hepatitis is proposed.

Previous Investigations. As far as Australia is concerned, detailed investigation into the braxy-like diseases of sheep has been confined to a few workers.

In 1910 Dr. J. A. Gilruth (4) published the results of his researches into the etiology of a disease occurring in Victoria and Tasmania, thought by him to be the same in both States, although the organism which he considered the etiological factor differed slightly in the two cases. In Victoria and Tasmania the disease occurred only in the dry weather of summer, practically ceasing after the onset of the general autumnal rains. It was confined to young sheep (2–2½ years) in good condition, males and females being affected alike. The symptoms were rarely observed, and were confined to a general dullness and disinclination to move. As a general rule, however, animals were found dead early in the morning, after being apparently normal the preceding night. The post-mortem appearances were considered characteristic. Patches of gelatinous subcutaneous oedema, sometimes blood-tinged; serous cavities containing a variable quantity of clear or blood-stained watery exudate; stomach (abomasum) more or less deeply congested, but as a rule not ulcerated; liver showing mottled areas of necrosis; spleen slightly enlarged and softer than normal, kidneys congested, and lungs oedematous; sometimes subendocardial haemorrhages were present; lymph-glands more or less oedematous and haemorrhagic.

He states that "in animals dead for some hours the characteristic bacilli are present in the effusions, organs, or even in the blood, but never in a state of purity. They are most numerous in the peritoneal effusion, but invariably associated with other organisms—some long, thin, and filamentous, others broad and large, both sporulating, and probably of intestinal origin and putrefactive, while cocci may also be present." He points out that the peritoneal effusion is

very likely to be contaminated with putrefactive organisms if examined some time after death, on account of the sheep's carcase forming an excellent incubator for organisms, owing to insulation by wool and subcutaneous fat, thus enabling the bacterial flora of the large intestine to traverse the intestinal wall and reach the peritoneal fluid.

Gilruth attempted to purify his cultures by repeated passage through small laboratory animals or through sheep. This suggests that he considered one pathogen to be present in the original material. Such fluids as the peritoneal effusion of a sheep dead only a short time would be most likely to contain such pathogenic organisms as *Vibrion septique*, *C. sporogenes*, etc., and although there is no reason to doubt that the original pathogen could gain the peritoneal fluid, together with other bacteria, yet it is extremely likely that even after passage through guinea-pigs, the resultant material would contain a mixture of organisms. It is even possible for the original pathogen to be lost by passage. For instance, the organism isolated by the writer does not as a rule enter the blood-stream. If a culture of this organism contaminated with *Vibrion septique* be inoculated into a guinea-pig, and the heart blood cultured at the time of death, the result would almost certainly be a pure culture of *Vibrion septique*. It is highly probable that Gilruth was working with impure cultures, and assuming that the causal organism was present in the original material, it is possible that it may have been lost during the subsequent experiments.

It is interesting to note here that apart from the bacterial content of the peritoneal fluid and the wall of the abomasum, Gilruth's cases correspond very closely to those examined by the writer during the last two years (see later).

In March, 1921, Dr. S. Dodd (2) published an article on "Black Disease" in New South Wales. He concluded, after the examination of one hundred cases of this disease (all examinations being made on sheep killed while sick, or post-mortemed immediately death had taken place), that the primary lesion was an hepatic necrosis, and that all other lesions were secondary, and due to the action of the toxin produced by the bacilli in the liver and circulated in the blood-stream. He definitely determined that at the time of death organisms are confined to the hepatic lesions, and cannot be found elsewhere in the body, although in rare cases bacilli were cultured from the liver when no necrotic foci could be found. These he considered mass infections. He found the mucosa of the abomasum frequently congested, often in patches round the pylorus; infrequently circular ulcers of a superficial character were present in the mucosa of the abomasum, but these were sterile, and according to Dodd, of a peptic nature and a secondary character.

Another important observation made by Dodd was that all cases were infected with the liver-fluke *(Fasciola hepatica)*, and he advanced the theory that the liver-fluke is probably the mechanical carrier of the causal organism to the liver. Facts supporting this theory are the seasonal incidence of the disease, the geographical features of the infected paddocks, and the universal occurrence of

fluke infection in cases of "black disease." In New South Wales the disease is seasonal, occurring from late summer to early winter. Dodd suggested that black disease is probably identical with the braxy-like diseases of Victoria and Tasmania.

In Western Australia a disease of sheep occurs which simulates braxy, and which is known locally as "Beverley disease." It is confined to a fertile region in one of the oldest cultivated areas of the State. It has not been recorded from the wetter and colder parts, and is unknown in the long, dry summer, when feed is scarce, but commences in the autumn, with the first growth of herbage after the autumnal rains. Small outbreaks are the rule in winter months, adjacent farms being frequently simultaneously affected, but the first big outbreaks occur in the spring, "at the time of the spring transitional period." The disease is seasonal, resembling braxy in Europe. According to Dakin (1) sudden and unsuspected death is typical. He describes two types of symptoms, one characterized by unconsciousness and quiet death, the other by convulsions, head retraction, with death in 2–3 up to 24 hours, or even after several days. In many cases there is evidence of the sheep having scoured. The carcase is invariably that of a well-nourished sheep.

Post-mortem appearances described are mainly negative. The kidneys "present a marked lesion, being quite soft, sometimes almost fluid." There is little or no trace of inflammation of the intestines, and "certainly no necrosis of the mucous membrane." Lambs seem to be mainly affected, although losses amongst two-tooth sheep are common, and older sheep have suffered on some properties. Dakin concludes that the local disease in Western Australia is braxy, but that the organism supposed to be the cause in Scotland, Ireland, and Iceland was not the cause in Western Australia. Furthermore, in sheep examined immediately after death, *no organism could be discovered which could be regarded as the cause of the disease.* However, he states that "there was evidence that the disease was of bacterial origin, but the condition of the feed, and the treatment of the sheep in regard to it, seemed to be associated factors of great importance." No mention is made of the liver, examined either macroscopically or microscopically; the only bacteriological examination mentioned was of peritoneal exudate, which was found to be sterile.

Disease in Victoria. Two outbreaks of the disease in sheep were investigated by the present writer, one in 1921 and the other in 1922. These outbreaks occurred on two farms in the Goulburn Valley district in Victoria, where, judging by the history of the disease as elicited from the local farmers, it had been endemic for several years prior to 1921.

Occurrence. In both years (1921 and 1922) the deaths commenced in the late summer, and continued until the first heavy rains of winter (which in 1921-1922 did not occur until July and August in this district). No deaths occurred in the spring and early summer.

Symptoms. As a rule death is quite sudden, and the period during which symptoms are shown is so short as to be missed unless specially looked for. Sick sheep may be detected, as they lag behind the rest of the flock, being the last to rise when moved and the first to lie down. Later they assume a recumbent attitude, breathing becomes stertorous, and death usually follows within half an hour.

Post-mortem Appearances. Unless sheep are examined immediately after death putrefactive changes tend to mislead, and a false description of lesions is likely to result. Subcutaneous vessels may be injected—excess of peritoneal fluid, either clear or slightly blood-stained. Intestinal vessels injected—mucosa of the abomasum slightly reddened, but no sign of ulceration, gall bladder distended with bile. Pericardial sac distended with a clear fluid, coagulating on exposure to air, lungs and kidneys congested.

Liver shows the presence of irregular areas of necrosis, the necrotic tissue yellowish, grey, and friable. In at least two cases no trace of fluke infection could be detected.

Bacteriology. Cultures made from necrotic liver tissue were positive, that is, bacterial growths were obtained from them. All other cultures were negative, both aerobically and anaerobically. Anaerobic cultures of necrotic liver furnished a growth of a bacillus, apparently in pure culture.

Cultural Characters of the Bacillus. The original cultures were sown into bullock's heart medium (cooked meat medium). In this the organisms grow rather slowly, no gas formation being noticed as a rule earlier than 24 hours' incubation; 24–48 hours shows the commencement of gas formation, with slight reddening of the meat at the base of the tube. The medium is rendered acid. Cooked liver medium (using minced sheep's liver instead of bullock's heart), to which 2% peptone is added, produces the best growth for this organism. With this medium gas formation is usually marked at 18 hours, and a more vigorous and plentiful growth takes place.

The morphology of the organism varies according to the reaction of the medium and the rate of growth. In a rapidly growing culture, sown into cooked liver medium, titrated to about pH 7·2, the organism appears as a short, stout rod, with distinctly rounded ends, occurring singly, in pairs, or in short chains of three or four elements. In a slowly growing culture the individual elements are longer. As a rule sporulation does not begin until after 18 hours' incubation, although in some cases numerous sporulating forms will be seen if an 18-hour-old culture is examined. In media whose reaction is about pH 6·0 the bacilli appear longer, more slender, with a tendency to filamentous growth (up to 30μ in length), and show no tendency to sporulate.

Spores. These are large, distinctly oval, only slightly distend the bacillus, and are situated either terminally or subterminally. An occasional central spore has been observed. The addition of 2% peptone to the media seems to favour sporulation.

Staining. The organism is Gram-positive in early cultures, and *in situ* in the liver of natural cases of the disease. It rapidly loses the power of retaining the Gentian violet. A marked peculiarity of the bacillus is the beaded or vacuolated appearance of the organisms when stained by any method. This has no relation to sporulation.

Motility. The bacillus is non-motile on the slide, in a hanging-drop preparation.

All cultures degenerate quickly; this is especially seen in a rapidly growing culture. Examined after 48 hours, a culture will show mainly spores and ghost forms.

Broth. Unless every precaution is taken to ensure the medium being strictly anaerobic, cultures cannot be grown in broth: 2% peptone liver broth under vaseline has been used with moderate success. Tubes should be cooled rapidly after they are removed from the autoclave, and inoculated immediately. If left more than two days before being inoculated sufficient oxygen is absorbed from the air to prevent growth, and it seems impossible to drive this off by simple boiling. In addition care must be taken to use broth of a pH 7·6. (In inoculating broth under vaseline the following method was found to be satisfactory: Pasteur pipettes were constricted about ½ inch from the slender tip; sufficient inoculum was then taken from the seed culture, the pipette was pushed through the vaseline cap, and the tip of the pipette broken off by gentle pressure against the side or bottom of the tube. The hole in the vaseline cap left after the withdrawal of the pipette was then closed by heating the culture tube at the level of the vaseline until the latter began to melt. The melted paraffin then flowed and closed up the hole. The tube was then placed in cold water to hasten the solidification of the vaseline. A similar method was adopted to withdraw culture for microscopical examination.)

The growth in broth appears as a uniform cloudiness in 18 hours. Clearing and auto-agglutination commences after this, and is usually complete by the end of the second day. As a rule no gas is formed, though on one or two occasions a few gas bubbles have appeared after a little broth has been withdrawn for examination. No spores have ever been seen in broth cultures.

Agar: Surface Culture. On no occasion have I been able to grow this bacillus as a surface culture. Different media, such as blood agar, serum agar, and glucose agar, have been tried in McIntosh and Fildes' jar, and with pyrogallic acid, but with no sign of growth. Whether this is due to imperfect anaerobiosis or to deficiency in the media it is impossible to say.

Shake Culture. In 3% glucose agar growth has occasionally been accomplished. The colonies form in the depths of the media as woolly growths without a nucleus. No gas formation or shattering of the media is evident.

Brain Medium. The growth in this medium is slow; no blackening occurs, and only sluggish sporulation.

Alkaline Egg Medium, Serum Water, Sugar Media (except 3% glucose agar). No growth.

The organism appears to be an anaerobe of the strictest type, with fastidious reaction requirements.

Pathogenicity. The organism is extremely pathogenic for guinea-pigs, rabbits, and sheep. The effect on other animals has not been tested.

Guinea-pigs. Subcutaneous injection of 0·05 cc. of an 18-hour broth culture kills a guinea-pig in 24–36 hours. Slightly larger doses of meat cultures are necessary to kill in the same time. Several hours after inoculation the guinea-pig shows general malaise, and on examination a large swelling on the inoculated side can be felt. This usually extends from the inoculated groin forward, in some cases to the axilla of the same side, or even extending over the whole abdominal surface. The post-mortem appearances are typical. Extending from the point of inoculation forwards, the subcutaneous tissues are engorged with a watery oedema, slightly blood-tinged in the region of the inoculation point, but clear elsewhere. This exudate coagulates on exposure to air. Sometimes the subcutaneous tissues are nearly half an inch in thickness. There is no gas formation or emphysema or odour. The muscle of the leg may be paler than normal.

The intestines, especially the small intestines, show congested blood-vessels and general reddening, but there is no increase in the amount of peritoneal fluid; the spleen is normal. The liver may show one or two areas of necrosis under the capsule which are sterile and of toxic origin, and with few exceptions the gall bladder is found distended with bile. The organs of the thorax are normal, although there may be an increase in the amount of pericardial fluid. Cultures can usually be recovered from the subcutaneous oedema at the point of inoculation, but frequently those from a distance prove sterile. Smears of the subcutaneous exudate show very few organisms. Sometimes several fields are examined before one is seen. Other body fluids are sterile.

Sheep. Inoculation of 0·25 cc. or more of culture kills sheep in 36–48 hours. (Smaller doses have not been tried.) At 24 hours they show a definite lameness in the injected leg, which is swollen and painful. Later rumination ceases, the animal assumes a recumbent attitude, and dies quietly. Post-mortem appearances are on the whole similar to those of the guinea-pig. The oedema of the subcutaneous tissues is very extensive. No necrosis of the underlying muscles has been observed. In the abdomen there may be a slight excess of peritoneal fluid, the intestines are injected, and the mucosa of the abomasum shows congestion, which may be very marked. No necrosis of the liver has been observed, and the gall bladder is distended with bile. Both the liver and the kidneys are congested.

In the thorax, pericardium is distended with a pale clear fluid, which coagulates on exposure to air. The lungs and heart are normal. The heart-blood and fluids from the serous cavities prove sterile on culture. Organisms are not plentiful in the subcutaneous oedema, but can be recovered by culture.

Rabbits. Post-mortem appearances differ in these animals in that the oedema is not so marked.

Immunization Experiments. At the outset it was hoped to discover some method of immunizing sheep against this disease, preferably a method having a maximum of immunizing power and a minimum of risk. We decided to try the efficacy of germ-free preparations of the organisms, such as culture filtrates and aggressins (cf. Blackleg), and also killed vaccines. It was decided to eliminate these methods as useless before trying the effect of preparations such as attenuated live cultures. Although in such cases the process of attenuation may be generally successful, yet the introduction of live spores into animals is always accompanied by a certain amount of risk, due to the fact that some individuals may have a lowered resistance, or that in spite of the process of attenuation some spores retain a sufficient degree of virulence to produce illness, or even death. This is exemplified with some anthrax vaccinations, where, although carelessness in the introduction of vaccine can be excluded, yet a sufficiently large percentage of mortality takes place among the vaccinated animals to discredit the use of the preparation. In this connection mention might be made of the method of immunization by the introduction of live culture by the digestive tract. Gilruth produced an active immunity against the organism which he isolated from sheep by this method. Although no serious effects are produced in the animals themselves as a result of this method, yet it is highly probable that when a sporing culture is used, organisms *would pass through the digestive tract,* and thus bring about a further contamination of pasture, the very thing to be avoided where natural infection probably takes place by the ingestion of pathogenic organisms along with the food.

Bearing in mind the success with which sheep and calves can be immunized against blackleg by a germ-free culture filtrate of *B. chauvoei,* we decided to try the efficacy of a similar product in immunizing against the experimental disease produced by inoculation of the organism into guinea-pigs.

Two series of experiments were conducted in order to test the efficacy of culture filtrate as an immunizing agent. The medium used for the production of the filtrates was cooked liver medium, the second series differing from the first in that in the former 2% peptone was added to the medium.

Cultures of the organism were incubated for varying lengths of time, ranging from 2–14 days, then filtered, firstly through cotton-wool and filter-paper, and finally through a Chamberland F. candle. In some cases the culture was diluted before filtration. In each case the filtrate was tested for sterility before use. Six culture filtrates were prepared, but from the experiments conducted with them it was evident that no immunity could be established by the inoculation of doses of 1–2 cc. into guinea-pigs.

Vaccines Killed by Heat. Eighteen-hour broth cultures of the organism were centrifuged, and the organisms so deposited were emulsified in sterile saline and

subjected to a heat of 63°C. for 30 minutes, such a heat being sufficient to kill the vegetative forms of the organisms. Such vaccines were used in varying doses, but no immunity could be induced by the use of these preparations.

Aggressin Experiments. Two sheep were inoculated with 1 cc. of a cooked liver medium culture of the organism. *Expl. Sheep 5* was killed while moribund; *Expl. Sheep 7* was allowed to die, death taking place in 36 hours. The subcutaneous exudate in the inoculated thigh was collected as cleanly as possible and placed in sterile jars. This fluid partly coagulated on standing. It was then filtered through cotton-wool, the fluid from the coagulum being expressed by pressure. The filtrate was then passed through a Chamberland F. candle and put into amber-coloured sterile bottles. This germ-free filtrate proved very toxic on inoculation into guinea-pigs.

From Expl. Sheep No. 5 0·075 cc. was found to be the M.L.D. for guinea-pigs of about 500 grm. weight.

From Expl. Sheep No. 7 0·03 cc. was found to be the M.L.D.

Inoculations of 0·005 cc. into guinea-pigs produced marked symptoms and large swellings at the site of inoculation, but recovery resulted when such doses were used. No immunity resulted from inoculations with quantities under the M.L.D. (This was also the case when guinea-pigs recovered from an injection of live whole cultures.)

Toxin Production.

The toxicity of this fluid obtained from the sheep led to experiments to determine if under any conditions cultures could be obtained which on filtration would prove as toxic as this fluid. Previous experiments with culture filtrates showed that in one culture incubated for 48 hours the filtrate was toxic enough to produce death in doses of 1·5 cc.; incubation for periods longer than 48 hours yielded a non-toxic filtrate. (This is to say in doses of 1–1·5 cc.)

Filtrates were made of two cultures, one in broth, the other in peptone liver medium, after incubation for 18 hours. Both these filtrates proved toxic in doses approximating to the M.L.D. of the exudate from sheep, the filtrate from the broth culture proving the more toxic of the two. With the filtered exudate from sheep, and the filtrate from the 18-hour cultures, the post-mortem appearances of inoculated guinea-pigs are identical, and show a marked similarity to the condition produced in these animals by the inoculation of whole culture, the difference being the sterile nature of all fluids of the body. The gelatinous subcutaneous oedema is well marked, there is slight injection of the subperitoneal vessels of the intestine, the gall bladder is usually distended, and sometimes necrotic areas are present in the liver. Experiments were then undertaken to produce an "anti-toxin" to the toxin formed in the cultures and to the toxic filtrate of the exudate from sheep. The first series of experiments proved

fruitless. Guinea-pigs were inoculated with very small doses of toxin, and the doses were increased slightly every 7 days. It was found, however, that when the minimum lethal dose was reached the guinea-pigs succumbed, having derived no immunity from the inoculation of smaller doses. Further experiments on antitoxin production proved negative.

General Discussion of Experimental and Field Work. Although only a few cases of the natural disease have been investigated, there is no doubt that sheep in Victoria are affected with a condition resembling closely, if not identical with, black disease described by Dodd. Whether the disease has been introduced from New South Wales it is impossible to state, but there was no evidence of the sheep on either property having been brought from New South Wales, nor had sheep ever come from this source, as far as the owners could remember.

Judging from the post-mortem findings alone, the diseases would appear to be identical, the lesion in each case being an hepatic necrosis caused by an anaerobic bacillus.

Dodd considers that the liver-fluke is the mechanical carrier of the causal organism from the bowel to the liver. Recent work on the life history of the liver-fluke shows that the common route of the young fluke from the intestine to the liver is *via* the intestinal wall and peritoneal cavity, entering the liver through the peritoneal coat, and thence to the smaller bile-ducts. It is possible also that some may gain the liver by the portal blood-stream. What Dodd means by the "mechanical" carrier is hard to say, except on the supposition that young flukes enter the liver *via* the bile ducts. With our present knowledge of the method of infection by liver-flukes, one would expect to find peritoneal infections as well as liver infections if the fluke is the carrier of the organism. This is never found in natural cases. If it be a fact that black disease and fluke infection go hand in hand, another explanation of the relation of the liver fluke to the disease is suggested. It is possible that the presence of the parasites in the liver, with the consequent destruction of hepatic tissue, may produce a suitable nidus for the growth of the organisms which have gained the portal circulation, and any condition which has a deleterious effect on the liver cells, at the same time lowering the resistance of the tissues, would probably lead to infection, e.g., other migrating parasites, such as *Cysticercus tennicollis*.

The disease described by Gilruth is apparently the same as that which is present in New South Wales and Victoria at the present time. Apart from the bacterial content of the body fluids, which can be considered the result of post-mortem invasion, the necrosis of the liver stands out as the main lesion; in fact, by omitting from Gilruth's description of cases those conditions which can be ascribed to post-mortem changes, the similarity is remarkable, amounting almost to proof of identity.

As Gaiger (3) has definitely determined that braxy is a disease of sheep characterized by a bacterial necrosis of the mucosa of the abomasum, the term

braxy should rightly be used for such a disease, and no other. In the past all diseases of sheep which have had the one point in common, namely, a sudden and quiet death, have been grouped together as braxy, and hence much confusion now exists with regard to these diseases.

The Australian disease is seasonal, and the seasons of its occurrence are apparently the same both in Victoria and New South Wales, i.e., at the end of the summer, being limited or checked by the onset of the autumnal rains. It occurs when the country is dry and feed is scarce. During such times the close-cropping of sheep in their endeavour to find food is accompanied by the ingestion of a quantity of soil, and the appearance of the disease may possibly be explained in this way. With the onset of good rains, and a consequent luxuriant growth of grass, the possibility of ingestion of soil becomes more remote, and the disease slackens off or disappears altogether until the following summer. The organism isolated by Dodd bears a strong resemblance to the organism isolated by the writer in Victoria, the main difference being the extreme difficulty with which the latter organism is cultivated. Dodd has produced cultures in milk, alkaline egg medium, and brain broth, and also stab cultures in agar, whereas no cultures have been obtained in these media by the writer. Also Dodd found liquid paraffin adequate on the surface of his media in preserving anaerobic conditions, but this material was found to be useless in the case of the Victorian organism.

Histology of the Liver Lesions. The lesions in the liver of affected sheep have been in each case histologically identical. These consist of an irregular necrosis of hepatic tissue. The shape and appearance of the lobules of the liver are maintained in the necrotic area, but the individual cells are smaller, non-nucleated, and irregular. The necrotic tissue is bounded by an irregular zone of tissue reaction. This is made up of a dense mass of leucocytes, both small-celled and poly-nuclear types being present. Immediately inside this zone can be seen the bacilli associated with the lesion. They are present in large numbers, the majority lying immediately adjacent to the zone of reaction, but others are scattered throughout the necrotic tissue. The bacilli appear singly, in pairs, and in fairly long filaments, and here and there a sporing element can be seen. Outside the reaction zone the liver capillaries are engorged with blood, and the liver cells show cloudy swelling. Away from the lesion the liver is congested, but otherwise shows no apparent change in histology.

In addition to these necrotic areas in the liver, in several cases examined other pathological processes have been seen, which apparently bear no relation to the necrotic process. They consist of areas of leucocytic aggregation, composed largely of eosinophiles, the remainder being polymorphonuclear leucocytes. Exactly what the nature of these foci is has not been determined, but the high eosinophile content would suggest that they were parasitic in origin, and probably associated with fluke infestation.

The organism isolated from natural cases of the disease in Victoria has been compared with other anaerobes in this laboratory, but it cannot be considered to resemble any of these very closely. There are, however, some points of resemblance between it and *B. oedematiens* (Weinberg), as described by different American workers. It differs, however, in point of size and in the reluctance with which it grows in many of the stock types of media.

SUMMARY.

1. There is present in Victoria a disease of sheep similar to black disease as described by Dodd, and distinct from braxy of Scotland.

2. The main and primary lesion is an hepatic necrosis, due to infection with an anaerobic bacillus.

3. The method of natural infection is unknown.

4. The bacillus isolated in Victoria resembles *B. oedematiens* (Weinberg) in a number of its characteristics.

5. Recovery from artificial infection with the Victorian organism produces no immunity.

6. Aggressin and culture filtrate have no immunizing power.

7. The organism produces a toxin in 18-hour-old culture filtrates, which decreases on further incubation, and is virtually absent after three days.

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